Journal of Fish Diseases 2009, 32, 893–900 doi:10.1111/j.1365-2761.2009.01068.x

Exophiala xenobiotica infection in cultured striped jack, *Pseudocaranx dentex* (Bloch & Schneider), in Japan

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Abstract

This report describes *Exophiala* infection in cultured striped jack, *Pseudocaranx dentex*, in Japan in 2005. One hundred out of 35 000 fish died per day and mortalities continued for 1 month. Diseased fish showed swelling of the abdomen and kidney distension. Numerous septate hyphae, pale brown in colour, were seen in kidney in squash preparations. Histology revealed abundant fungal hyphae and conidia in gill, heart and kidney. Fungal hyphae were accompanied by cell necrosis and influx of inflammatory, mainly mononuclear cells. The fungus isolated from the diseased fish had septate hyphae, pale brown in colour and 1.8–3.0 μm in diameter. Conidiogenous cells were conspicuous annellides, short or cylindrical or fusiform in shape. Conidia were one-celled, ellipsoidal with smooth walls, accumulated in balls at the apices of annellides that tended to slide down, 1.5–2.0 μm in width and 3.0–5.0 μm in length. The fungus was classified into the genus *Exophiala* based on its morphology and as *Exophiala xenobiotica* based on the sequences of the ITS 1–5.8S–ITS 2 regions of rDNA. This is the first record of this fungus in a marine fish.

Keywords: Exophiala xenobiotica, histopathology, Japan, *Pseudocaranx dentex*, striped jack.

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Introduction

Fungal infection caused by the genus *Exophiala*, known as black yeast, has been reported in several species of fish. The first report was by Carmichael (1966) who described a systemic infection of cutthroat trout, *Salmo clarki* Richardson, and lake trout, *Salvelinus namaycush* (Walbaum). The diseased fish exhibited cranial ulcers and erratic swimming. The causative agent was initially named a *Phialophora*-like fungus but later classified as *Exophiala salmonis*. Later, Fijan (1969) reported a systemic mycosis in channel catfish, *Ictalurus punctatus* (Rafinesque), with skin ulceration and numerous nodules in all internal organs. The aetiologic agent was identified as a *Phialophora*-like fungus but later reidentified as *E. pisciphilus* (McGinnis & Ajello 1974). There are subsequent reports on *Exophiala* infection in many other fish such as Atlantic salmon, *Salmo salar* L. caused by *E. salmonis* (Richards, Holliman & Helgason 1978; Otis & Wolke 1985), cod, *Gadus morhua* L., by an *Exophiala*-like fungus (Blazer & Wolke 1979), *E. werneckii* from spoiled salted freshwater fish (Mok, Castelo & Barreto Da Silva 1981), *Exophiala* sp. nov. from Atlantic salmon (Langvad, Pedersen & Engjom 1985), smooth dogfish, *Mustelus canis* Mitchill, by *E. pisciphila* (Gaskins & Cheung 1986), Atlantic salmon by *E. pisciphila* (Langdon & McDonald 1987) and *E. psychrophila* (Pedersen & Langvad 1989) and King George whiting, *Sillaginodes punctata* Cuvier, by *Exophiala* sp. (Reuter, Hutchinson, Ham & Davis 2003). *Exophiala jeanselmei* was isolated from eastern box turtle, *Terrapene carolino carolina* L. (Joyner, Shreve,

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Spahr, Fountain & Sleeman 2006). *Exophiala* species have also been found in various environments, such as *E. jeanselmei* isolated from soil and wood (Dixon & Shadomy 1980), *E. alcalophila*, *E. dermatitidis*, *E. jeanselmei* and *E. moniliae* from bathwater and sludge (Nishimura, Miyaji, Taguchi & Tanaka 1987), *E. angulospora* from well water (Iwatsu, Udagawa & Takase 1991), *E. mesophila* from dental unit waterlines (Porteous, Grooters, Redding, Thompson, Rinaldi, De Hoog & Sutton 2003) and *E. xenobiotica* from habitats rich in monoaromatic hydrocarbons and alkanes (De Hoog, Zeng & Harrak 2006). In this study, we described the first case of *E. xenobiotica* infection in cultured striped jack, *Pseudocaranx dentex* (Bloch & Schneider), characterized by morphological, biological and molecular studies.

Materials and methods

A fungal infection occurred in cultured young striped jack in April 2005. The cumulative mortality was approximately 8.6% within 1 month. Five of 100 moribund fish (on average 5 cm in body length and 2 g in body weight) were randomly collected for examination.

Fungal isolation

Kidneys from diseased fish were aseptically collected and inoculated onto PYGS agar (peptone 1.25 g, yeast extract 1.25 g, glucose 3 g, artificial sea water 37.60 g, agar 12 g) for 10 days. One conidium was taken from each culture plate and inoculated onto PDA (Nissui Co. Ltd) to obtain a pure culture. All isolates were identified as the same fungus from their morphological characteristics.

Fungal identification

For identification of isolates, the shape and colour of colonies were first observed after 1-month incubation at 25°C on PDA and slide cultures were made of isolates grown on PDA and mounted in lactophenol cotton blue. Identification was according to De Hoog, Gené & Figueras (2000). For scanning electron microscopy, 5-mm^2 agar blocks with growing mycelia were fixed in 2.5% glutaraldehyde for 24 h at 4°C and post-fixed in 1% osmium tetroxide for 10–20 h at 4°C. They were then dehydrated in ascending ethanol grades, finally submerged in *t*-butanol, freeze dried (JEOL JFD-310) and coated with palladium and gold (JEOL, JFC-1600). Samples were examined with a scanning electron microscope (JEOL, JSM-6380 LV). The effect of temperature and NaCl on growth of isolates was examined following the method of Munchan, Kurata, Hatai, Hashiba, Nakaoka & Kawakami (2006).

Molecular biological identification based on ITS 1–5.8S–ITS 2 regions of rDNA

The sequences of the ITS 1–5.8S–ITS 2 regions of the rDNA were obtained by routine methods. Briefly, DNA was extracted with a DEXPAT® kit (TaKaRa) using a modified procedure. Approximately 100 μL of fungal cells cultured at 25°C for 2 months on potato dextrose agar slants were placed in a sterilized microtube (1.5 mL); then 0.5 mL of DEXPAT® solution was added and the mixture was homogenized with a plastic pestle. It was then incubated at 100°C for 10 min and centrifuged at 13 201 ***g*** for 10 min. The supernatants were used as DNA samples. Although this type of kit is designed for extracting DNA from paraffin-embedded tissue samples, we routinely use it for the isolation of genes from fungal cultures because of its convenience (Murata, Sano, Ueda, Inomata, Takayama, Poonwan, Nanthawan, Mikami, Miyaji, Nishimura & Kamei 2007). We mixed 2.5 μL of the DNA extract with Ready-to-Go beads (Amersham Pharmacia), 2.5 μL of 10 pM of the primers ITS-5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (Swofford 2001) and 17.5 μL of distilled water. The reaction mixture was subjected to one cycle of denaturation at 95°C for 4 min, 30 cycles of amplification at 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, and a final extension cycle at 72°C for 10 min with a PCR thermal cycler MP (TaKaRa). The PCR products were confirmed by electrophoresis in 1.0% agarose in 1 × TBE buffer (0.04 M Tris-boric acid, 0.001 M EDTA, pH 8.0) followed by ethidium bromide staining. The PCR samples were purified using a PCR purification kit (QIAquick®; Qiagen Co. Ltd.) and labelled with BigDye® Terminator Ver. 1.1 (Applied Biosystems) following the manufacturer's protocol. The labelled samples were directly sequenced on an ABI PRISM® 3100 sequencer (Applied Biosystems) using the primers ITS-5, ITS-4, ITS-2 (5′-GCT GCG TTC TTC ATC GAT GC-3′) and ITS3 (5′-GCA TCG ATG

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AAG AAC GCA GC-3′) (White, Bruns & Lee 1990). DNA sequences were aligned using GENETEX-MAC genetic information processing software (Software Development Co. Ltd).

One hundred and three nucleotide sequences from *Exophiala* spp. obtained from the GenBank database, together with the sequences obtained in this study, were aligned using the computer program CLUSTALX (Version 1.8) (Jeanmougin & Thompson 1998), followed by manual adjustments with a text editor. Phylogenetic analyses were performed with PAUP v4.0b10 (Swofford 2001), using a heuristic search for maximum parsimony trees. Basepair composition and transition and/or transversion patterns of the data set were estimated by PAUP v4.0b10. Bootstrap values were calculated over 1000 replicates to assess branch topology. A phylogenetic tree was selected from 1000 unrooted trees and was drawn by Tree View PPC (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Clades were supported by bootstrap values above 90%.

Histopathology

The gill, heart and kidney of diseased fish were fixed in 10% phosphate-buffered formalin solution. Samples were routinely embedded in paraffin and sectioned at 5 μm. The sections were stained with haematoxylin and eosin (H&E) and Grocott's variation counter stained with haematoxylin and eosin (Grocott's-H&E).

Results

Diseased fish showed clinical signs at necropsy such as abdominal swelling (Fig. 1) and kidney distention. Microscopic examination of the kidney of diseased fish revealed numerous septate hyphae, pale brown in colour, in squash preparations.

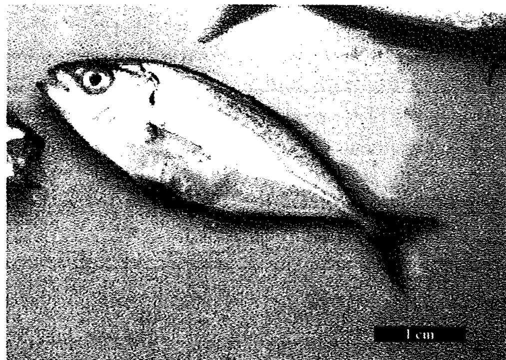

Figure 1 A diseased striped jack showing swelling of abdomen.

Fungal identification

The colony morphology of the fungus after 1 week of incubation on PDA at 25°C was initially a black yeast form. It then became woolly and velvety and olive brown in colour but black on the reverse side after 4-week incubation (Fig. 2). Hyphae were septate and pale brown in colour, 1.8–3.0 μm in diameter. Conidiogenous cells were conspicuous annellides, short, cylindrical or fusiform in shape. Conidia were one-celled, ellipsoidal with smooth walls and accumulated in balls at the apices of annellides that tended to slide down, 1.5–2.0 μm in width and 3.0–5.0 μm in length (Fig. 3). Colony radius of the fungus incubated on PDA at 10, 15, 20, 25, 30 and 35°C was 1.1, 8.6, 18.3, 23.3, 24.8 and 0 mm, respectively. Colony radius of fungus incubated on PDA including 0%, 1%, 3%, 5% and 7% NaCl was 23.8, 23.7, 19.7, 13.2 and 3.1 mm, respectively. The fungus grew poorly at 9% NaCl and there was no growth at 11% NaCl.

Molecular identification based on the ITS 1–5.8S–ITS 2 regions of rDNA

One of the 1000 most-parsimonious trees obtained from heuristic searches based on the 103 sequences of the ITS1/2 region is shown in Fig. 4. The tree consists of seven clades and was rooted to *Cladophialophora carrionii* (AB109169). Clade I consists of *Exophiala spinifera* and *E. nishimurae*, II is made up of *E. oligosperma*, *E. jeanselmei* and *Exophiala* sp., III is of *E. jeanselmei*, IV is of *Exophiala* sp., *E. spinifera*, *E. xenobiotica* and the present isolate, V consists of *E. dermatitidis*, VI of *E. heteromorph* and VII of *Exophiala* sp., *E. lecanii-corni*, *E. mesophila*, *E. salmonis* and *E. pisciphila*. *Exophiala crusticola* appears as an orphan sequence.

Histopathology

Numerous fungal hyphae were found in gill, heart and kidney of striped jack. Ellipsoidal conidia were particularly observed in the lesions (Fig. 5). The presence of fungal hyphae was accompanied by cell necrosis and an influx of inflammatory, mainly

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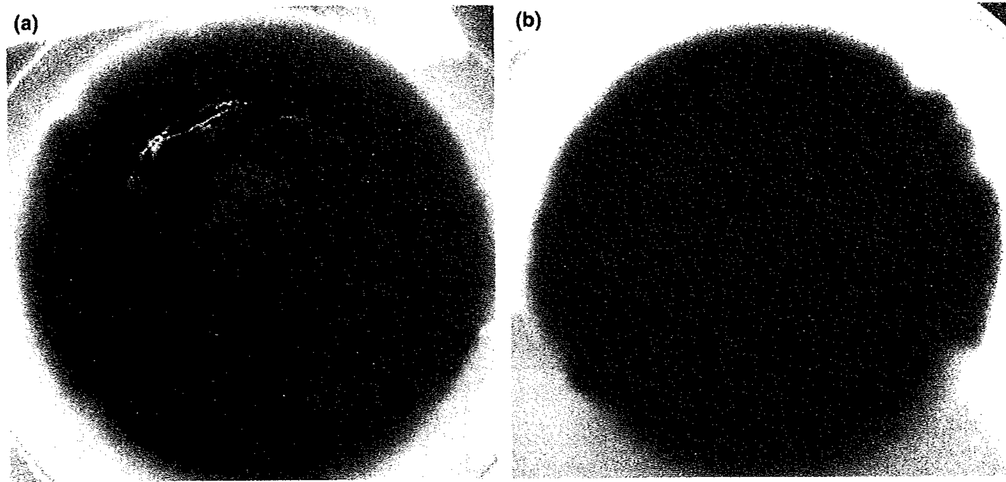

Figure 2 Colony of *Exophiala xenobiotica* cultured on PDA at 25°C for 4 weeks. (a) Surface is initially moist, becomes woolly and velvety with age, olive brown in colour. (b) Reverse side is black.

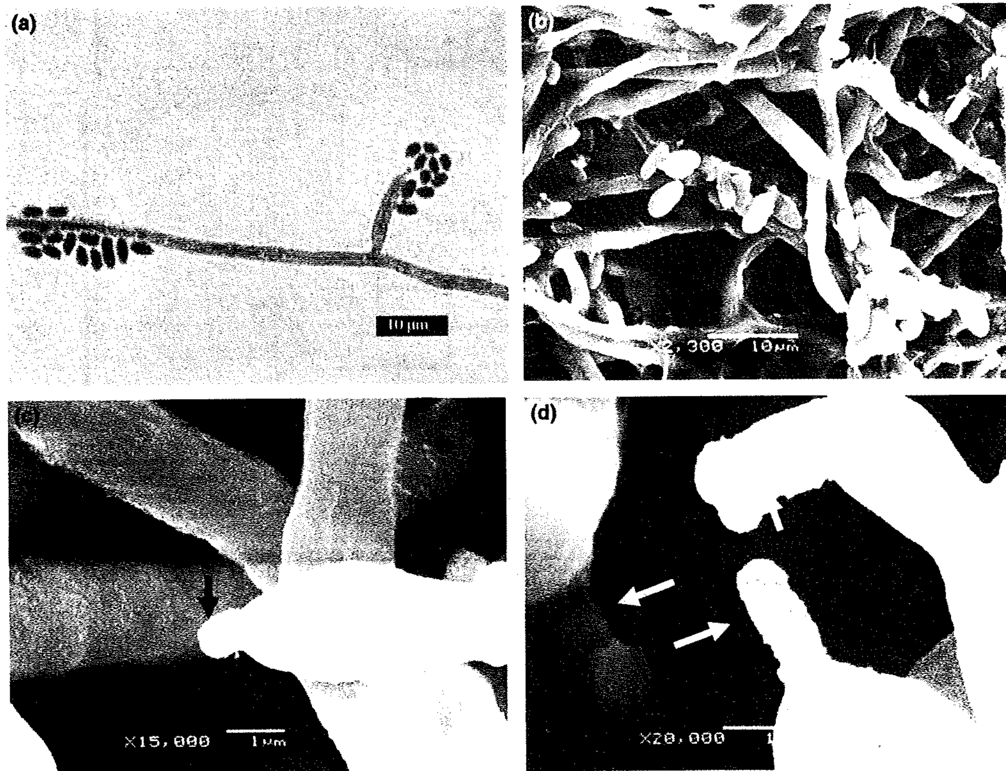

Figure 3 *Exophiala xenobiotica.* (a) Slide culture on PDA after 4-week incubation at 25°C (lactophenol cotton blue). Conidiogenous cell with conidia accumulated in balls at apices and tending to slide down. (b–d) Scanning electron photomicrographs. (b) One-celled conidia, ellipsoidal with smooth walls. (c–d) Conspicuous annellides conidiophores (arrows).

mononuclear cells. Mats of fungal hyphae were embedded at the base of gill filaments. Lamellar fusion and hyperplasia were associated with the fungal hyphae. Granulomatous inflammation was seldom found. The centre of granulomas consisted of eosinophilic necrotic parenchyma and fungal hyphae.

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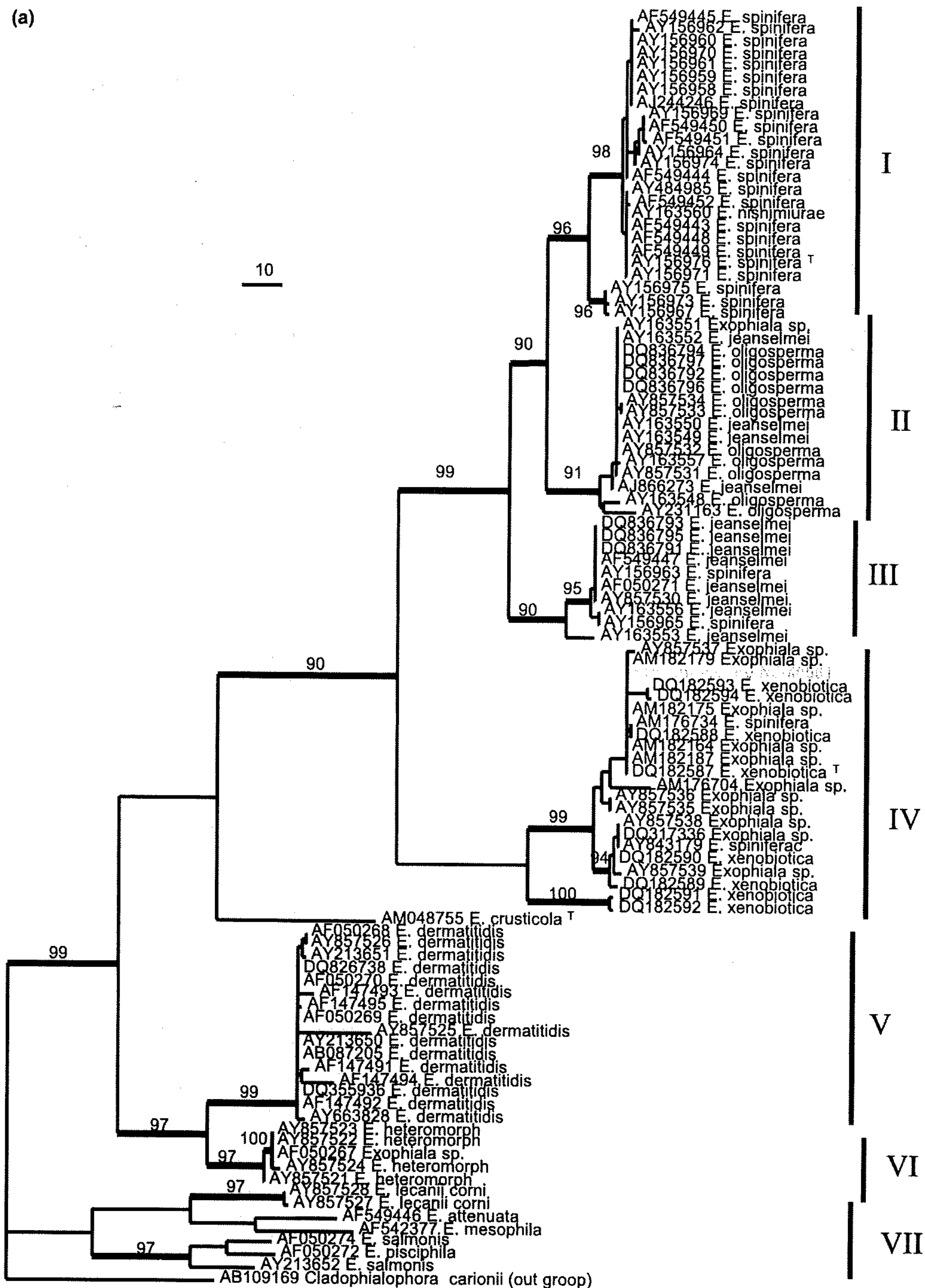

Figure 4 Most-parsimonious tree based on 103 sequences of the ITS 1–5.8S–ITS 2 regions of ribosomal RNA. The scale bar shows 10 changes; bootstrap support values (> 90%) from 1000 replicates are shown at the nodes. Thickened lines indicate the restrict consensus branches. The tree was rooted to *Cladophialophora carionii* (AB109169). (a) Clade I consists of *Exophiala spinifera* and *E. nishimurae*, II of *E. oligosperma*, *E. jeanselmei* and *Exophiala* sp., III of *E. jeanselmei*, IV of *Exophiala* sp., *E. spinifera*, *E. xenobiotica* and the present isolate, V of *E. dermatitidis*, VI of *E. heteromorph* and VII of *Exophiala* sp., *E. lecanii-corni*, *E. mesophila*, *E. salmonis* and *E. pisciphila*. (b) A close up image of the clade IV.

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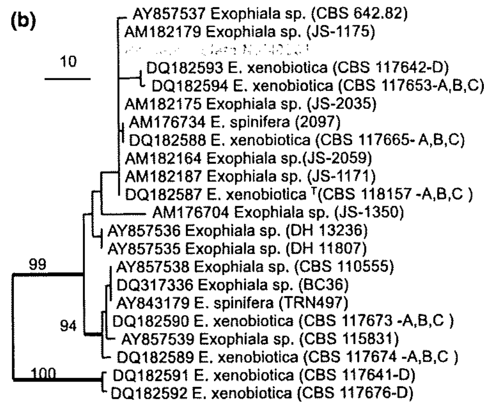

Figure 4 Continued.

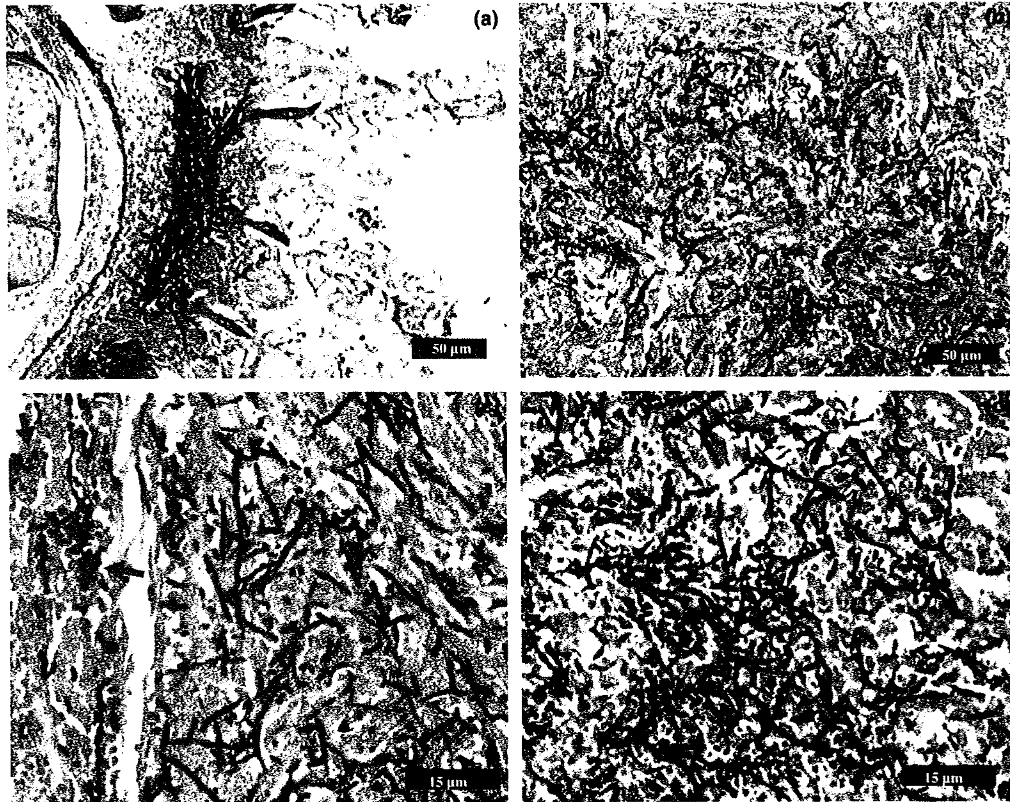

Figure 5 Histopathological features of *Exophiala xenobiotica* infection in striped jack. (a) Gill showing mats of fungal hyphae embedded in gill arch and penetrating into gill lamellae. (b) Cross-section of the heart showing heavy infiltration of fungal hyphae. (c) Higher magnification of heart tissue showing conidial apparatus (arrows). (d) Fungal hyphae invading kidney associated with necrosis and influx of inflammatory cells (a–d = Grocott's-H&E).

Discussion

Exophiala spp. are called black yeast fungi because they produce a black yeast phase on culture media. The genus has so far been reported as pathogenic in fish or other aquatic animal populations (Carmichael 1966; Fijan 1969; Richards *et al.* 1978; Blazer & Wolke 1979; Langvad *et al.* 1985; Otis & Wolke

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1985; Gaskins & Cheung 1986; Langdon & McDonald 1987; Pedersen & Langvad 1989; Reuter *et al.* 2003; Joyner *et al.* 2006). The species of *Exophiala* reported in fish include *E. pisciphila*, *E. psychrophila* and *E. salmonis*.

The morphology of the present isolate, NJM 0561, differed from the three species of *Exophiala* previously found in fish in the conidial size and in growth temperature (Pedersen & Langvad 1989). However, these criteria are of limited value for distinguishing between these species. All morphological and biological characteristics, together with molecular data, are necessary to identify a fungus. Isolate NJM 0561 was similar to *E. xenobiotica* described by De Hoog *et al.* (2006) in colony appearance, conidiogenous cell shape and conidia shape, but our isolate did not produce chlamydospores and failed to grow at 35°C. The isolate did grow at 10–30°C and in 1–9% NaCl and appears to be able to survive in a broad range of environmental conditions. In phylogenetic analysis of the ITS1-5.8S-ITS2 regions, the present isolate, which was isolated from the kidney of marine fish, corresponded with the source of the other isolates in clade 4 which originated from sea water. However, Uijthof, Figge & De Hoog (1997) constructed a phylogenetic tree based on the ITS1 sequence of *Exophiala* species described from fish, including *E. pisciphila* and *E. salmonis* and found they formed a heterogeneous group. The present study used not only ITS1 but also the 5.8S and ITS2 regions and found our isolate separated from other fish pathogenic *Exophiala* species. The present fungus was identified as *Exophiala xenobiotica*, based on the presence of annellidic conidiogenesis to identify genus and molecular analysis of the ITS1-ITS2 regions for species identification.

Clinical signs of the diseased fish included swelling of the abdomen and kidney distension, as reported by previous authors (Richards *et al.* 1978; Langvad *et al.* 1985; Otis & Wolke 1985). However, there was no ulceration or any other skin lesions in the present case, which contrasts with the findings of Fijan (1969), Blazer & Wolke (1979), Otis & Wolke (1985), Gaskins & Cheung (1986), Langdon & McDonald (1987) and Reuter *et al.* (2003). Histopathology revealed abundant fungal hyphae in the gills, heart and kidney of diseased fish. The parenchymal cells of the affected organs which were invaded by hyphae showed prominent necrosis and there was mononuclear cell infiltration. Neither granulocytes nor giant cells were found in contrast to the previous reports (Fijan 1969; Richards *et al.* 1978; Blazer & Wolke 1979). The route of transmission of the fungus in the present case is unknown, however, the presence of fungal hyphae in the gills may indicate that the infective stage might pass through the gills into the fish circulatory system. This report is the first record of striped jack infected by *E. xenobiotica* in Japan.

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Received: 22 November 2007
Revision received: 20 February 2008
Accepted: 27 March 2008

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Journal of Clinical Laboratory Analysis 23:139–143 (2009)

Detection of *Paracoccidioides brasiliensis* gp43 Gene in Sputa by Loop-Mediated Isothermal Amplification Method

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The fungus *Paracoccidioides brasiliensis* is the pathogen of paracoccidioidomycosis (PCM), a systemic mycosis prevalent in Latin America. The loop-mediated isothermal amplification method (LAMP) was used in this study to detect the presence of *P. brasiliensis* in sputa samples from patients with chronic PCM, suspected PCM, and a negative control. The target *P. brasiliensis* gp43 gene was amplified in less than 4 hr in 11 of 18 sputa samples tested. The LAMP method had the advantage of speed and simplicity compared with the classic diagnostic methods such as the histopathological test or biological material culture and did not require sophisticated technical apparatus. It would be an important aid in cases where immediate treatment would mean patient survival, especially in immune-suppressed patients. J. Clin. Lab. Anal. 23:139–143, 2009. © 2009 Wiley-Liss, Inc.

Key words: gp43 gene; *Paracoccidioides brasiliensis*; paracoccidiomycosis; LAMP

INTRODUCTION

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the *Paracoccidiodes brasiliensis* (*P. brasiliensis*) fungus. To date, the natural habitat and saprophytic life conditions of *P. brasiliensis* have not been totally clarified (1). There are reports of isolation from penguin feces from Antarctica (2), soil samples (3), dog meal, soil-contaminated (4), fructivore bat gut (5), and armadillo gut (6).

The geographic distribution of the mycosis is restricted to Latin American countries (5,7). As it is not a disease with obligatory notification, its true prevalence cannot be calculated. In endemic countries, such as Brazil, it is estimated that the annual rate of incidence in the population is 1–3 per 100,000 inhabitants and mortality of 0.14 per 100,000 inhabitants (8). It is estimated that in these regions there are approximately 10 million persons infected by *P. brasiliensis* and most have no clinical symptoms (1).

The PCM infection mechanism occurs by conidia installation, which can be destroyed in the lung parenchyma or produce a focus of infection drained to the lymph node region, forming the primary infection complex. It can disseminate by the hematogenic and/or lymphatic path, reaching other organs and causing the juvenile or acute form (9) or remain with a primary scar focus with viable fungi called a "quiescent lesion," which evolves to chronic PCM (10).

Diagnosis is based on culture, histopathology, and antibody detection in clinical samples. The latter can be problematic depending on the antigen used, with the occurrence of cross-reaction, and false-positive or false-negative results (11). In cases of patients with immune problems, although it is not considered an opportunist fungus, immunological diagnosis can be hindered by reduced antibody production. In histological sections, *P. brasiliensis* might be missed or confused with other dimorphic fungi such as *Histoplasma* spp. or *Coccidioides immitis* (12).

In non-endemic areas, diagnosis is problematic for clinicians, pathologists, and microbiologists unfamiliar

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Received 16 March 2008; Accepted 8 January 2009
DOI 10.1002/jcla.20304
Published online in Wiley InterScience (www.interscience.wiley.com).

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with PCM and its etiological agent (13). To improve the sensitivity and specificity of PCM diagnosis, *P. brasiliensis* identification has been attempted by molecular techniques. Recently the LAMP technique was used by Endo et al. (14) to identify the species-specific gene gp43 of *P. brasiliensis* without cross-reaction with other fungus isolates. Using only one sample of histological cuts, it was suggested that the method could be used in other types of clinical samples.

The LAMP method was assessed in this study for PCM diagnosis in human sputa samples using the same primers that were used by Endo et al. (14). It is a fast and specific alternative method; the use of sputa is a non-invasive sample collection technique and does not expose the patients to risks. It can be used as a complementary technique especially in cases where a delay in diagnosis may put patients at risk.

MATERIALS AND METHODS

Clinical Sputa Specimens

The sputa samples obtained from 17 patients with chronic PCM (male, aged 28–72 years) with a diagnosis established by X-ray, clinical data, and isolation by culture and/or serological tests (Table 1, samples 2–16 and 18–19), one suspected PCM (Table 1, sample 17) and 01 negative PCM control (Table 1, sample 20) from the Pneumology and Infectious Disease Sector, University Hospital, State University of Londrina, Londrina, Paraná, and Sanitary Pneumology Sector of Integral Infection-contagious Center, Municipal Health Service, Londrina, Paraná were collected in Brazil between November 2000 and June 2003. The backgrounds are shown in Table 1 (Samples 2–20). The sputa were defrosted and fixed with 70% ethanol overnight, dried in Brazil at room temperature, and analyzed in Japan. The dried sputa were washed with distilled water three times with centrifuging, and processed for DNA extraction.

A 44-year-old male who was 3rd generation of Japanese-descendant Brazilian was diagnosed with PCM by clinical manifestation, cytological observation of the sputum, and fungal culture in Japan (15). The preliminary study for detection of gp43 of *P. brasiliensis* from the sputum by the LAMP method was positive (Table 1, sample 1). The sputum was fixed with 70% ethanol for overnight, rinsed with distilled water three times, and processed for DNA extraction.

Extraction of DNA From Sputa

DNA was extracted with DEXPAT® (TaKaRa Biomedical, Inc., Ohtsu, Japan) according to the manufacturer's instructions. Five hundred microliters of the extract solution was added to the washed pellet of sputum, whose volume was approximately 50 µl. The mixture was incubated at 100°C for 10 min and

TABLE 1. Patient's backgrounds and results of diagnoses

				Immunological test				
Sample number	Age	Involvement	Chest X-ray	ELISA	ID	Cytology or biopsy	Fungal isolation	LAMP
1	44	P	+	ND	ND	+	+	+
2	65	M	+	1/800	1/16	+	+	−
3	46	M	+	1/400	1/4	+	−	−
4	45	MP	+	1/200	1	+	−	+
5	50	M	+	1/400	1/8	+	−	+
6	72	P	+	1/800	1/16	−	−	−
7	53	P	+	1/400	−	−	−	+
8	65	L	+	1/200	1/8	−	−	+
9	46	M	+	1/200	−	+	−	+
10	67	M	+	1/800	1/8	−	−	+
11	70	P	+	1/800	−	+	−	+
12	52	P	+	1/800	1/16	+	−	−
13	51	M	+	1/800	1/4	+	−	−
14	41	P	+	1/200	1/4	−	−	+
15	50	M	+	1/400	−	−	−	+
16	50	M	+	1/800	1	+	+	−
17	70	P	+	1/800	−	−	−	−
18	40	P	+	1/200	−	−	−	+
19	77	P	+	1/800	1	−	−	−
20	28	Control	−	−	−	ND	ND	−

All patients are male. P = pulmonary involvement, M = mucocutaneous involvement, and L = lynphatic involvements (ND = not done; − = negative result). 1 = Patient diagnosed in Japan, 2–19 = patient diagnosed in Brazil, and 20 = healthy control.

centrifuged at 12,000 rpm (13,201 *g*) for 10 min. The supernatant was then processed for the LAMP.

LAMP Method

The LAMP method used in this study was developed by Endo et al. (14) and detects the gp43 gene with a combination of F3 for LAMP, B3, FIP, and BIP primers designed from the partial sequence of gp43 (GenBank accession number U26160) by a registration system primer designing web site (FUJITSU Ltd., Tokyo, Japan: "LAMP PIMER EXPLORER" web site in "Netlaboratory" homepage http://venus.netlaboratory.com/partner/lamp/index.html). These primers recognize an area of gp43 where variation among strains has not been reported.

The primer sequences were as follows: F3 for LAMP, 5-TCA CGT CGC ATC TCA CAT TG-3 used in the species-specific forward primer; B3, 5-AAG CGC CTT GTC CAA ATA GTC GA-3 used in the species-specific reverse one; FIP, 5′-TGG CTC CAG CAA TAG CCA CCC GTC AAG CAG GAT CAG CAA T-3′ designed from the forward sequence of 425th–445th and the complementary sequence of 464th–485th; and BIP: 5′-CAT GTC AGG ATC CCG ATC GGG CCT TGT ACA TAT GGC TCT CCC T-3′ designed from the forward sequence from 648th to 668th and the complementary sequence from 691st to 712th. One microliter of 10 ng/ml DNA template, and 40 pmol each of the FIP and BIP primers, and 5 pmol each of the F3 for LAMP and B3 primers were mixed with 12.5 μl of 2 reaction mix in the kit (Loop AMP, Eiken Chemical Co., Ltd., Tokyo, Japan) in a final volume of 23.0 μl. DNA mixtures were incubated at 63°C for 120 min. The reaction was stopped by heating the mixture at 80°C for 2 min to inactivate the enzyme of LAMP amplification. The amplified products were electrophoresed in 1.0% agarose gels in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA) and stained with ethidium bromide. DNA extracted from a *P. brasiliensis* isolate (IFM 41621, Pb-18) was used as a positive control for the amplification.

Double Immunodiffusion in Gel

Six glass slides (2.5 × 7.5 cm) were covered with a 3.0 mm-thick layer of agar (1% agar, 0.85% NaCl, and 0.02% sodium azide) with seven wells (one central and six peripheral). The *P. brasiliensis* exoantigen sample was applied to the central well, and serum sample in duplicate with no dilution, and 1:2–1:32 dilutions were applied to the peripheral wells. The samples were incubated in a humid chamber at room temperature for 24 hr. The gel was washed, dried, and stained with amido black.

RESULTS

LAMP-amplified products appeared as a ladder of bands on electrophoretic gels. The preliminary trial of the sputum from a Japanese patient is shown in Figure 1(a). It was possible to amplify the target gene in 11 out of 18 sputa samples from Brazilian PCM (Fig. 1: (b, c)).

LAMP-positive samples included cases that were negative by cytology/biopsy and fungal isolation (Table 1). Without correlation to LAMP, 12 out of 18 patients were positive in immunodiffusion (ID) test for PCM. LAMP-negative samples included 8 samples that are positive by ELISA (Table 1 samples 2, 3, 6, 12, 13, 16, and 19), 7 samples positive by ID (Table 1 samples 2, 3, 6, 12, 13, 16, and 19), by biopsy (Table 1 samples 2, 3, 12, 13, and 16) and by biological material culture (Table 1 samples 2 and 16).

DISCUSSION

There is difference in sensitivity and specificity for immunological test to confirm PCM diagnosis. Morphological and physiological characteristics used in classic diagnostic methods are complex. Developing simpler and complementary techniques has become important, especially due to the increase in the number of immune-suppressed patients. Therefore, a clear diagnosis of fungal infections is necessary.

The certainty diagnosis of PCM consists in showing the pathogen in histological preparations, visualization in fresh or in culture exams (16). However, there are cases where physical or clinical state prevents access to the lesion because invasive methods are usually used for collection. In these cases, serological techniques provide important auxiliary diagnosis. Although the serological techniques such as ID and ELISA are used to confirm PCM diagnosis, the rates of false-positive and false-negative results are still very high, and the specificity and sensitivity of the technique are directly related to the antigen or antibody used (17).

ID was the method used by clinical laboratories because it is an easy procedure, but the results can vary because of different parameters, including the antigen preparation, the form of the disease, and the starting of treatment (18). The follow-up of patients receiving treatment for PCM has shown that the antibody titers obtained by the ID test frequently do not correlate with the clinical status of the patient (19). In some patients, high antibody titers were observed until the end of the treatment when the patients were clinically cured. On the other hand, low antibody titers are related to the absence of clinical symptoms in most patients. However, in some cases low titers are present although clinical symptoms are present (20).

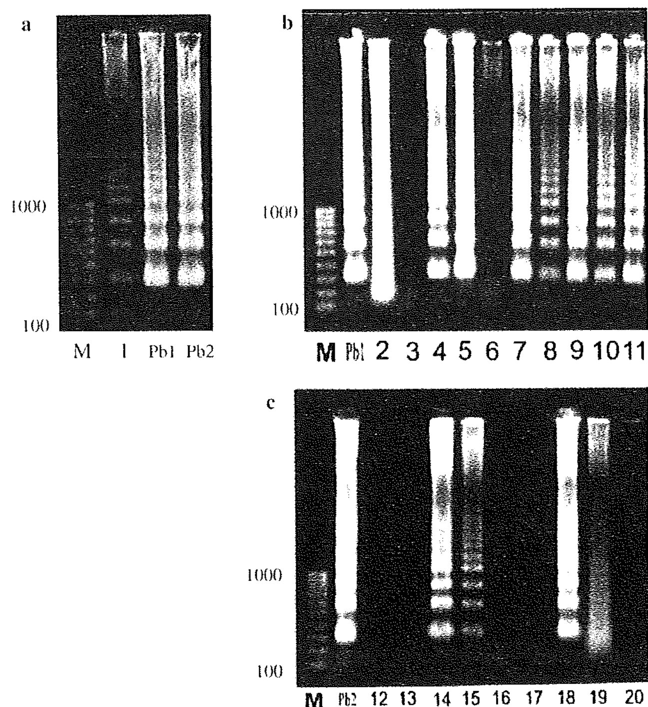

Fig. 1. The detection of gp43 gene of *P. brasiliensis* by the LAMP method. **(a)** 1 = First PCM case diagnosed in Japan by LAMP; **(b, c)** 2–19 = Brazilian PCM patients and 20 = Healthy control (Table 1). The specific DNA banding patterns for the gp43 gene of *P. brasiliensis*: Pb1 = IFM 41621, Pb2 = Pb 18, and M = marker.

The ELISA test has been used to detect antibodies in most systemic mycosis. In spite of this, compared with the immune diagnosis of PCM, the technique still offers high percentages of crossed reactivity, especially in patients with histoplasmosis, candidiasis, "Jorge Lobo" disease, and recently against sera from apparently healthy persons, resident in PCM endemic areas (11).

Against other molecular tests, LAMP has shown the advantage of greater sensitivity, because using clinical samples and the same target *P. brasiliensis* DNA, a minimum of 1 ng was detected by the PCR technique (21), whereas in LAMP, the minimum sensitivity reported was 100 fg (14). Moreover, LAMP does not require sophisticated technical apparatus, neither high biosafety.

In the first instance, the results presented here can be interpreted as false negatives or with low sensitivity because from 18 potentially positive samples, 11 were confirmed. However, when analyzed case-by-case, we can state that the LAMP method was successful in the proposed task.

The negative sample by LAMP number 17 (Table 1) was from a patient who presented a non-specific lung clinical condition, later diagnosed as candidiasis, which explained the ELISA reagent by a crossed reaction. This assertion was supported by the non-demonstration of *P. brasiliensis* on the histopathological tests and negative attempt of isolation by culture.

Most samples (Table 1 samples 2, 3, 6, 13, and 16), before collecting the sputa samples, received medication treatment with sulfametoxazol and trimetoprima, which explained the non-presence of the fungus in sputa but in the tissues confirmed by the highly reactive ELISA (majority with 1/800, except for sample 3) and the positive isolation in two biopsy tissue samples (Table 1, samples 2 and 16).

In group of negative LAMP, the form classified as lung (Table 1, sample 6) was negative for culture and for visualization of the fungus by histopathology, which was in fact, a sample from a patient considered cured. Two cases were of tuberculosis, where there was no *P. brasiliensis* isolation (Table 1 samples 12 and 19), only visualizing on a histopathology slide (Table 1 sample 12). This would be expected because the lesions can remain surrounded by tissue for years in lesions with viable fungi, which does not mean active fungemia (17). There is a possibility that, at the moment when the sputa samples were collected from this group, except on sample number 3 where the etiological agent was different, there was a PCM infection. The positive radiological result in these cases are due to so-called "radiological lung scars" (12).

On the other hand, the 11 positive samples were of PCM disease, in its chronic form, with lesions in the oropharinge, in the process of acute clinical condition. All these patients were from centers of reference for long-term PCM treatment, with an average of 10 years treatment, with previous diagnosis demonstration by biopsy or culture. Medication treatment was quickly established, which led to clinical improvement and normalization of serological tests (data not shown). Through assertive treatment, the results by culture were all negative. This supports the need for other techniques for *P. brasiliensis* detection, because although it is considered the diagnostic gold standard, the small trace of fungus found in the biopsy tissue can lead to non-isolation (9, 11, 17).

Thus, the LAMP method can complement PCM diagnosis. It has the advantage that cross-reactivity does not occur. It is fast, sensitive, and false-positive results do not occur because it uses a single diagnostic parameter. It is the detection of the single nucleotide sequence for *P. brasiliensis*, unlike some immunological tests that use indirect detection methodology of micro-organisms, which depends on many unknown factors of parasite–host relationship for certain diagnosis.

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話題の感染症

ヒストプラズマ症の最新の知見 ―家庭内飼育動物が罹患したら―

Histoplasmosis **: Un update**

佐野文子（さの あやこ）
Ayako SANO

要旨

ヒストプラズマ症はコクシジオイデス症、パラコクシジオイデス症、ブラストミセス症、マルネッフェイ型ペニシリウム症とならぶ輸入真菌症の一つとして取り扱われてきた。しかし、国内感染例がヒト、イヌ、ウマで確認されていることから、わが国も流行地に含まれると考えられるようになり、最近では、ヒストプラズマ症を高度病原性真菌症として扱われるようになってきた。

原因菌は真菌（カビ）の*Histoplasma capsulatum*で、温度依存性の二形性真菌である。特徴的な大分生子と球形から涙滴型の小分生子を形成する。

感染は主に、小分生子を吸入することにより起こるが、創傷からの感染もある。

症状は無症状から軽い感冒症状、肺炎が主な症状であるが、全身感染に至った場合、肝臓や脾臓などの細網内皮系器官の腫脹、副腎、骨髄、脳などを侵し、免疫低下を伴う基礎疾患がある場合は重篤になりやすい。病理組織学的には細胞内寄生性の小型の酵母細胞として検出される。一方、わが国のイヌでは皮膚症状を主徴とし、難治性の潰瘍が特徴である。

最近、海外渡航歴の無いヒトおよびイヌ症例由来の原因菌の遺伝子型は、戦前、ウマで流行していたヒストプラズマ症の一種の仮性皮疽と近縁であることが示唆されていることから、わが国で土着のヒストプラズマ症はウマの仮性皮疽の異種寄生であると考えられている。重要なことはウマの仮性皮疽は接触感染が主な感染経路である。現在までに、感染したイヌとの接触により二次感染した例は無いが、一般家庭での罹患動物の飼育は安全管理および経済的に問題となるため、安楽死も考慮しなければならない。

はじめに

ヒストプラズマ症は、高度病原性真菌症の一つで、コクシジオイデス症、パラコクシジオイデス症、ブラストミセス症、マルネッフェイ型ペニシリウム症と同様に輸入真菌症の一つとして取り扱われてきた[1,2]。輸入真菌症とは本来日本に存在せず、海外の特定地域でのみ発生している高度病原性真菌症のうち、日本国内で発生したものをさす。ヒストプラズマ症は感染症予防法等で管理されている感染症ではないが、2008年末現在、ヒト58例（千葉大学真菌医学研究センター 輸入真菌症患者発生最新状況 http://www.pf.chiba-u.ac.jp/）、イヌ8頭（未発表1例を含む）[3-7]、ウマ1頭[8]（ただし、戦前、仮性皮疽として把握されている症例は約20,000頭）、ラッコ3頭[9]、ウシ4頭以上[10,11]が確認または報告されている。

ほとんどの真菌感染症は、抵抗力の低下したホストに感染する（日和見感染）のに対し、これらの高度病原性真菌症原因菌は、いずれも感染力が強く、しばしば健常人にも発生して深在性や全身性感染（deep-seated mycoses, systemic mycoses）を起こす。

表1に国立感染症研究所による主な病原真菌の危険度分類を示した。この表で危険度レベル3に属する真菌はすべて高度病原性真菌症の原因菌で、細菌でいうコレラ菌、チフス菌、ペスト菌、炭疽菌等と同レベルの高い感染性を示す。

真菌は一般に細菌、ウイルス、リケッチアなどに比べ病原性の強いものは少ない。しかし真菌は他の

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微生物とは大きく異なり、細胞の核に核膜をもつ真核微生物である。いったん感染、発症すると、抗真菌剤は真核細胞である宿主細胞に多大な副作用をもたらすため使用できる薬剤が限られ、全身感染を起した場合、治療が困難である。これが真菌症の恐ろしいところである。

なかでも、ヒストプラズマ症は、海外の流行地への渡航歴を持たずに国内で感染したと推測されている症例として、ヒト8例以上[12~19]、およびラッコを除くその他の動物症例全てが該当する（**表2**）。さらにわが国で発生しているイヌのヒストプラズマ症は、主な臨床症状がヒト症例と異なり、難治性の潰

表1　真菌の危険度分類

危険度	該当する真菌
レベル1	レベル2および3以外の菌
レベル2	*Aspergillus fumigatus* *Candida albicans* *Cladophialophora carrionii* *Cladophialophora bantiana* *Cryptococcus neoformans* *Exophiala dermatitidis* *Fonsecaea pedrosoi* *Sporothrix schenkii*
レベル3	*Blastomyces dermatitidis* *Coccidioides immitis*、 *Histoplasma capsulatum*（*H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*） *Histoplasma farciminosum*（*Histoplasma capsulatum* var. *farciminosum*） *Paracoccidioides brasiliensis* *Penicillium marneffei*

註：*Aspergillus* spp., *Chaetomium* spp., *Fusarium* spp., *Myrothecium* spp., *Penicillium* spp.の毒素産生株はレベル2扱いとする。

表2　国内で感染したと推測されるヒストプラズマ症

症例	年齢（歳）	性別	発病地	症状	診断方法	予後	文献
ヒト							
1	17	F	岡山	全身播種	H	死亡	(12) Acta Med Okayama 11 : 347-364, 1957.
2	24	M	熊本	呼吸器	H	不明	(13) 結核 36 : 194, 1961.
3	67[a]	M	新潟	全身播種	H	死亡	(14) 結核 59 : 256-257, 1984.
4	72	M	群馬	皮膚	C, CL, H	治癒	(15) J Dermatol 21 : 586-589, 1994.
5	84	M	大阪	全身播種	H, MB	死亡	(16) 日本病理学会会誌 93 : 387, 2004.
6	78	M	京都	全身播種	C, CL, H, MB	死亡	(17) Tropical Medicine and Health 33 : 40, 2005.
7	47	M	岡山	肺	H, MB	経過観察中	(18) 日本呼吸器外科学会雑 22 : 92-96, 2008.
8	59	M	奈良	全身播種	H, MB	経過観察中	(19) 感染症学会雑誌 82 : 588, 2008.
ウマ[b]							
1	4	F	栃木	全身播種	H, IA	死亡	(8) Jpn Vet Med Sci 63 : 1229-31, 2001.
ウシ[c]							
4	3カ月	F	岩手	全身播種	H	死亡	(10) Jpn J Vet Sci. 34 : 333-339, 1972.
イヌ							
1	8 : 雑種	F	東京	粘膜・皮膚	H, IH	治癒	(3) J Vet Med Sci. 60 : 863-5, 1998.
2	2.6 : MD	M	東京	皮膚	H, MB	治癒	(4) 真菌誌 42 : 229-35, 2001.
3	2.3 : SZ	F	熊本	皮膚	H, MB	緩解・増悪	(4) 真菌誌 42 : 229-35, 2001.
4	5 : 柴	F	東京	皮膚	H, MB	起立不能により安楽死	(5) Vet Microbiol, 94 : 219-24, 2003.
5	4 : SH	M	東京	皮膚	C, H, MB	緩解その後追跡不可能	(6) J Vet Med A 52 : 472-480, 2005.
6	12 : SZ[d]	M	東京	皮膚	C, MB	緩解・増悪	(7) Medical Mycology 43 : 233-245, 2007.
7	8 : BT	F	千葉	皮膚・全身播種	H, MB	死亡	(7) Medical Mycology 43 : 233-245, 2007.
8	13 : LR	F	千葉	肺	H, MB	死亡	未発表
ラッコ[e]							
1	4.75	F	新潟	全身播種	H, IH	死亡	(9) J Comp Pathol 125 : 219-23, 2001.

a；1984年までに岡山（1が相当すると思われる）、鹿児島、熊本（2が相当すると思われる）、長崎、久留米、福岡、大宮、新潟、山形など国内発症例13例（9例は病理組織、4例は臨床診断）が確認されていたと報告されている。b；この他に仮性皮疽として国内および外地の症例として20,000頭が記録されていた。c；明治時代に5例が記載されていた（時重獣医学博士論文集：214-216, 1918）。d；2005年2月老衰のため死亡。e；症例個体は日本で出生、親は輸入個体で、ヒストプラズマ症により死亡と推定されている。MD：ミニチュアダックスフント、SZ：シーズー、SH：シベリアンハスキー、BT：ボストンテリア、LR：ラブラドールレトリバー、C：細胞学的診断、CL：培養陽性、H：病理組織学的診断、MB：分子生物学的診断、IH：免疫組織学的診断。

瘍となり、さまざまな背景から、膿や侵出液の付着による接触感染の可能性が皆無とはいえず、家庭内で飼育管理する場合の安全性が問題となっている。

そこでヒストプラズマ症をわが国に存在する最も危険な真菌症と位置づけ、ヒトも動物も同じ感染源から感染する広義の人獣共通感染症（サプロズーノーシス：sapro zoonosis）の一つとして、獣医師の観点から紹介する。

Ⅰ. 原因菌

H. capsulatum は室温での発育が遅く、集落は粉状から綿毛状となる。初め白色で次第に黄褐色を帯びてくる。裏面は黄色あるいは黄橙色を呈する。顕微鏡的には分生子柄（conidiophore）および短い菌糸側枝の先端に大、小の分生子（conidium）が形成される。大分生子（macroconidium）は直径7～25μm、球形または西洋梨形である。細胞壁は厚く表面には多くの指状の突起がみられる。小分生子（microconidium）は直径2～6μm、球形あるいは西洋梨形である（**写真1**）。1%ブドウ糖を添加したブレイン・ハート・インフュージョン寒天（brain heart infusion agar）を用い、35～37℃で培養すると白

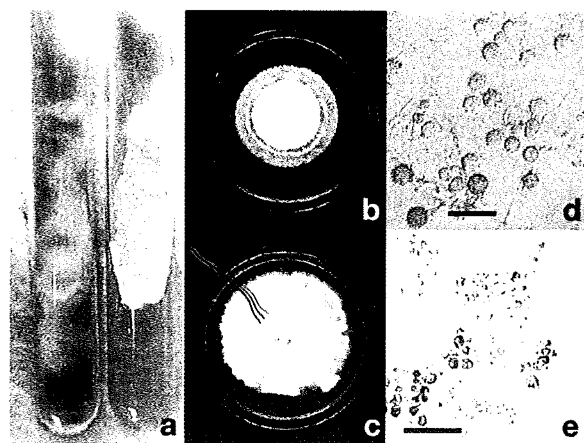

写真1 **a.** ポテトデキストロース寒天斜面培地、25℃、2カ月間（左）と1%ブドウ糖添加ブレインハートインフュージョン寒天斜面培地、35℃、7日間の酵母様集落（右）、**b.** サブロー培地 **c.** ポテトデキストロース寒天培地上での集落、25℃、2カ月間、**d.** ホルマリン固定したポテトデキストロース寒天斜面培地上の集落のかき取り標本、ラクトフェノールコトンブルー固定・染色、バーは20μm、x200、**e.** ホルマリン固定酵母様集落のかき取り標本ラクトフェノールコトンブルー固定・染色、バーは20μm、x400.

色～淡黄色の酵母様集落を形成する株もある。酵母細胞は球形または卵円形、直径2～4μmである[20]。しかし、一般検査室での形態学的観察は、高度病原性真菌であることから、推奨できない。

Ⅱ. 原因菌のvariety

3種類のvarietyに分けられていた[20～23]。現在、真菌のバーコード遺伝子といわれているリボゾームRNA遺伝子のITS領域の配列や、多遺伝子解析の結果、varietyに分ける概念は否定されている[24～26]。しかし、後述するわが国のイヌのヒストプラズマ症に関連する概念として、あえて紹介する。

H. capsulatum var. *capsulatum* によるカプスラーツム型ヒストプラズマ症（histoplasmosis capsulati）は全世界的に大河の流域を流行地とし、ヒトをはじめ各種動物が罹患する。自然界での本菌の棲息地はコウモリの糞やヒバリの巣が関連しているといわれている。単にヒストプラズマ症と呼ばれているものはこの病型である。わが国で発症した輸入症例のほとんどがこの病型である。

H. capsulatum var. *duboisii* によるズボアジ型ヒストプラズマ症（histoplasmosis duboisii）はアフリカを流行地とし[20,22]、わが国でも輸入症例が数例報告されている。一方、海外の報告では、1957年にYamatoらが報告した症例の病理組織像での酵母細胞の大きさがやや大型であったことから[12]、わが国にもこの型のヒストプラズマ症が固有に存在することが示唆されている[20,22]。ヒトの他にヒヒが罹患することが知られている。

H. capsulatum var. *farciminosum*（または *H. farciminosum*）によるファルシミノーズム型ヒストプラズマ症（histoplasmosis farciminosi）は全世界的に分布する[20,23]。ウマ、ロバ等の奇蹄獣に発症したときにこの病名が使われ、仮性皮疽、流行性リンパ管炎やウマカサなどの別名がある。

カプスラーツム型とズボアジ型との違いは後者がアフリカ大陸でみられ、感染組織内の酵母細胞が前者のそれに比べて大きく（直径8～15μm）、組織内に多数の巨細胞が出現してくるという以外は両菌種の間に菌学的（形態的）に差は認められない。またファルシミノーズム型はカプスラーツム型と形態学的な違いはない[20～23]。

Ⅲ. 分布

ヒストプラズマ症は世界各地、温帯、亜熱帯、熱帯に分布し、特に大河の流域が流行地である[20～23, 27, 28]。

カプスラーツム型の第1の流行地として米国のミシシッピー川流域があげられる。ヒトの症例はいうまでもなく、特にヒバリの巣から本菌が高頻度で分離されている。

第2の流行地はアマゾン川流域である。この地方のインディオには「洞窟に入ると風邪を引く」という言い伝えがあり、洞窟に棲息するコウモリの糞に棲息している*H. capsulatum*の分生子を吸入することにより発症する。わが国でもテレビ取材班がアマゾンの洞窟内の撮影を行ったとき、コウモリの糞に繁殖していた*H. capsulatum*の分生子を吸い込み8名全員が感染した例が報告されている。また、この地方に棲息する野生動物も本菌の保菌者として知られている。ラテンアメリカ諸国では、ブラジル、アルゼンチン、ベネズエラなどでの報告のほかに、グアテマラ、メキシコも流行地で、この地域は第1の流行地から連続した流行地と考えられる。

なお、わが国も土着症例がヒトと動物で報告されているが、コウモリの糞、土壌などから分離された記録は今のところない[29]。

第3の流行地として東南アジアがある。特にタイのメコン川流域が多発地域となっている。さらに中国、台湾、フィリピン、シンガポール、インドネシア、マレーシア、バングラデシュ、インド、オーストラリア、ニューカレドニアなどで症例が報告されており、オセアニアも含めて、東南アジアから東アジアに広域に分布していると考えられる。

第4の流行地として地中海沿岸があげられる。特にイタリアは土着の症例と外国帰りおよび外国人の症例が混在している。この地域の特色として、カプスラーツム型と次に述べるアフリカ型ヒストプラズマ症ともいうべき、ズボアジ型ヒストプラズマ症の双方が混在していることである。

ズボアジ型ヒストプラズマ症の流行地はアフリカである。わが国では輸入症例としてアフリカからの外国人渡航者に、ズボアジ型が確認されている。

ファルシミノーズム型ヒストプラズマ症の流行地としてエジプト、スーダン、インド、パキスタン、東欧諸国、旧ソビエト連邦、東南および東アジア各国および戦前のわが国などがあげられる[30]。

Ⅳ. 感染経路

呼吸器感染が主な感染経路である。しかし、ファルシミノーズム型ヒストプラズマ症で創傷感染、接触感染が飼育管理上問題となっているうえ、皮膚潰瘍部位に接触したハエやアブによる媒介も示唆されている。また海外では、イヌのヒストプラズマ症の感染経路として消化器感染が示唆されている[31]。

Ⅴ. ヒトでの臨床症状

カプスラーツム型およびズボアジ型ヒストプラズマ症は肺に初感染が始まる。多くは無症状あるいは軽い風邪の症状程度で良性に経過する。しかし稀には数週間にわたる微熱が続くこともあり、米国では結核と間違われることも多い。少数の患者において感染は進行し、全身、とくに肝、脾、リンパ節など細網内皮系組織に転移する。皮膚の結節性紅斑や脳への浸襲を伴うこともある。エイズ患者では死亡率が高い。臨床症状により以下のように分けられている[32]。

1. 急性肺ヒストプラズマ症

感染から数週間後に、倦怠感、中程度の発熱、漠然とした痛みといった、感冒様の症状でふつう発症するが、多くは自然治癒する。潜在的なこともあれば、呼吸困難、血痰、胸痛を呈すこともある。胸部X線では、肺－リンパ節の複合病変が見られる。両側性または一側性の肺門部アデノパシー、実質への浸潤、粟粒性または結節性の透亮像がいろいろな程度で播種性に認められる。

2. 慢性肺ヒストプラズマ症

内因性または外因性の再感染の結果生じる。臨床上、咳嗽、血痰、呼吸困難、発熱、全身状態の不安定な変動が認められる。X線では、いろいろな程度に広がった浸潤像、時には偽性腫瘍状、さまざまな空洞、結核に似た肉芽腫性病巣、縦隔線維症などを示す。経過は緩徐で、慢性の呼吸不全や肺性心となる。

3. 播種性ヒストプラズマ症

血行性、リンパ行性、網内系を介して病原菌が播種する結果生じる。熱発し、全身状態はひどく悪化する。リンパ節腫大、肝腫大、骨髄病変（貧血、白血球減少症）、中枢神経症状（髄膜炎、脳脊髄炎）、心病変（心筋の三層にわたる）と、肺または縦隔、骨、胃腸、皮膚粘膜、副腎（副腎不全）、腎、眼球に病変を作る。治療をしないと、本型は常に致命的である。本型は糖尿病、悪性血液疾患、ステロイド治療、後天性免疫不全症候群（AIDS）などの免疫不全の症例によく見られる。

4. その他

稀ではあるが口唇、舌、歯肉、鼻咽喉、喉頭の粘膜および皮膚に潰瘍、結節を生じることがある。消化管の病変により、下痢、下血を起こすことがある。

また、わが国で渡航歴の無い症例に肺病変を欠き、皮膚病変だけに限られていたヒストプラズマ症例も報告されている[15]。

Ⅵ. ウマの臨床症状

ウマ等で発症するファルシミノーズム型ヒストプラズマ症は頚部や脚のリンパ管やリンパ節を特異的に侵す。ヒト、ウシ、イヌ、ラクダでも発疹、潰瘍、肉芽腫形成など皮膚症状を示すことが知られている[33]。また重症では肺炎症状を伴うこともある。

Ⅶ. イヌの臨床症状

1. アメリカ合衆国での臨床症状

Rhoades[34]によれば 1.良性肺型（benign pulmonary form）ヒストプラズマ症と、2.全身性（disseminated form）ヒストプラズマ症の2型に分けられている。良性肺型は肺に小結節を形成し、慢性の咳嗽を症状とする。小結節は腫瘍の転移病巣と鑑別する必要がある。一方、全身性の場合、呼吸器症状は重篤な上、細網内皮系細胞にびまん的に寄生するため、各種リンパ節、肝臓、脾臓の腫脹を伴う。小腸、心臓、腎臓および皮膚に病変が及び予後不良である。

また Nielsen[35]によれば臨床型は 1. 急性、致死性、全身感染型（acute, fatal, disseminating disease）、2. 進行性慢性型（advanced chronic form）、3. 非致死型（non-fatal form）に分けられている。流行地におけるイヌのヒストプラズマ症の多くは全身性もしくは急性、致死性が多く、診断されても予後不良の症例が多い。

写真2　皮膚に潰瘍を生じたイヌのヒストプラズマ症の1例。

2. わが国のイヌでの臨床症状

日本で確認されたイヌのヒストプラズマ症は、皮膚潰瘍と粘膜の病巣を特徴とする（**写真2**）。基礎疾患に免疫不全があり、重篤な場合は肺病変を認める[7]。多発性皮膚結節を生じ、肺病変を欠く類似症例がオーストラリアで報告されている[36]。これらの皮膚症状は、*H. capsulatum* var. *farciminosum* によるウマの仮性皮疽との類似点である[2,4～7]。

Ⅷ. 診断方法

1. 培養検査

本菌種は高度病原性真菌であるから、実験室内感染には十分注意しなければならない。専門機関に相談することが第一である。喀痰、膿、生検材料よりの *H. capsulatum* の分離は当然のことながら、隔離された安全キャビネット内でマスク、帽子、手袋、ゴーグル、専用ガウンなどの防御装備を装着して行われなければならない。

本症は菌分離率が低いことが確定診断の妨げとなっている。室温で、ポテト・デキストロース斜面培地を使用し、4週間まで観察することを推奨する。可能ならば数本の培地を用いると、形態学的観察に

も使用できる。ただしシャーレは決して使用しない。真菌検査一般に使用されているサブロー培地および病原性酵母の呈色試験用培地は初代分離には推奨出来ない。

肉眼で観察できる集落が認められたら、安全キャビネット内で試験管のシリコ栓から注射針を通じて、培地を含めた最終濃度が70%になるようにエタノールを静かに注入し24時間以上固定したのち、リボゾームRNA遺伝子のinternal transcribed spacer (ITS) 1-5.8S-ITS 2領域の配列を決定して同定することが望ましい。

本菌の形態学的同定の決め手として、特徴的な大分生子の確認が提唱されている。しかし、一般的な真菌で行われている載せガラス培養は行ってはならない。培養後、数週間から数カ月経過後、実体顕微鏡下で試験管を観察し、大分生子の形成が確認できたら、培地の分量の約1/10量のホルマリン原液を試験管のシリコ栓から注射針を通じて注入し、24時間以上固定し、かきとり標本を作製する。ラクト・フェノール・コットンブルーで再度固定し染色すると観察しやすい。

また35～37℃における酵母様細胞の確認も有用であるが、分離株によっては酵母形への変換が困難なこともある。方法は微小な集落を確認した時に、集落表面が濡れる量の滅菌水を試験管のシリコ栓から注射針を通じて注入し、1%ブドウ糖添加ブレインハート・インフュージョン寒天斜面培地に植え替える。ただし熟練し、安全な無菌操作が行える場合に限られる。

2. 血清学的診断

血清中や尿から抗原を検出する方法と、抗体を検出する方法がある[32]。ラテックス凝集法、免疫拡散法、補体結合法などのキットが海外で販売されているが、日本での入手は数週間以上要するため、緊急の場合は専門機関に相談することを推奨する。

かつて行われていたヒストプラスミン反応は、補体結合反応による血清中の抗原検出の妨げになるうえ、播種性ヒストプラズマ症の患者では陰性となることもある[32]ため、2000年以降、発売中止となっている[37]。

ただし、国内感染ヒト症例[16]およびほとんどのイヌ症例は血清学的診断法で陰性を示すことが知られている[4~7]。海外でもイヌ症例での血清学的診断法は陰性を示すことがあると報告されている[31]。

3. 病理学的検査

病理組織では著しい肉芽腫性炎症反応が特徴的である。これら肉芽腫を形成している組織球は細胞性免疫不全の場合、取り込んだ酵母細胞を殺すことが出来ず、菌は組織球内で増え続ける。生検組織（骨髄も含む）、血液塗抹、肺洗浄液、皮膚病巣浸出液などがその材料となる。しかし、病理組織だけではマルネッフェイ型ペニシリウム症、クリプトコックス症、スポロトリコーシス、*Candida glabrata*感染などとの鑑別が重要である[32,33]ため、確定診断に至ることはできない。ここでは著者の都合により、イヌ症例の病理組織像を紹介する。

まず、ヘマトキシリン・エオジン染色ではマクロファージに取り込まれた菌体の周囲にハローを形成することが特徴である（**写真3**）。真菌を特異的に染色するPAS染色も菌体の確認には有用である（**写真4**）が、染色性が弱いものもあり、グロコット染色で菌体を確認することを推奨したい（**写真5**）。また、イヌの場合、潰瘍部の浸出液の塗抹をギムザ染色により観察すると、マクロファージ内に酵母細胞を確認できることもある（**写真6a-c**）。

4. 遺伝子診断

パラフィン包埋組織、生検組織、浸出液などからの遺伝子検出による診断が可能である。千葉大学真菌医学研究センターでは500塩基程度を決定し、遺伝子型の推定を試みている。

方法は菌体や臨床検体から抽出したDNAをnested-PCRにかけ、塩基配列を決定し、BLASTサーチ (http://blast.ncbi.nlm.nih.gov/Blast.cgi) とそこから得られる簡易系統樹を用いる方法を行っている。はじめITS-5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') とITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') によりファーストPCRを行い、このPCR産物を10－100倍に希釈して、HcAF (5'-CAC GCC GTG GGG GGC TGG GAG CCT-3'とHcCR (5'-ATG GTG GGC RGG AGC CGG CC-3'のプライマーで増幅させ、バンドを検出し、これらのプライマーと配列中に含まれるユニバーサルプライマーのITS-2 (5'-GCT GCG TTC TTC ATC GAT GC-3')と

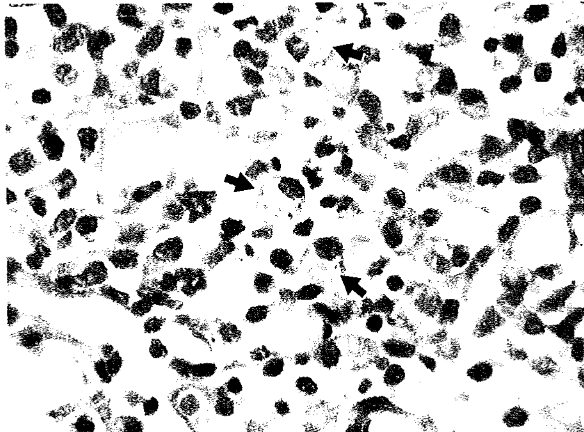

写真3 ヘマトキシリン・エオジン染色像：マクロファージに取り込まれた菌体（酵母細胞、矢印）の周囲のハロー（イヌ症例）、x400.

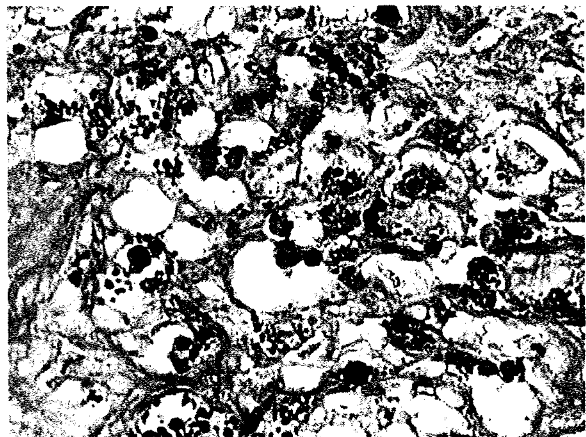

写真5 グロコット染色像、黒色に染め出された菌体、x400.

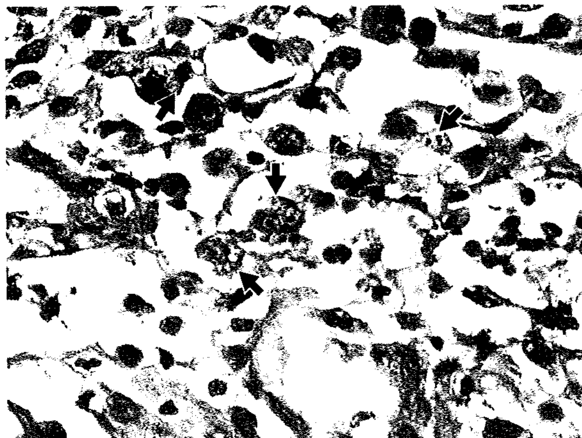

写真4 PAS染色像、赤紫色に染め出された菌体（矢印）、x400.

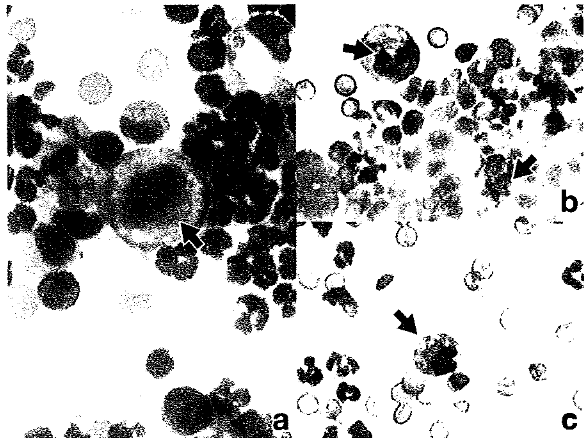

写真6 **a-c.** 潰瘍部の浸出液の塗抹で観察されたマクロファージ内の酵母細胞（矢印）、ギムザ染色、x1,000.

ITS-3（5'-GCA TCG ATG AAG AAC GCA GC-3'）によるによるラベル化により配列を決定している。PCR条件はいずれも95℃4分ののち、94℃1分、50℃1分半、72℃2分を40回、最後に72℃10分である[7]。

また、ヒストプラズマ症かその他の真菌症かを診断するには海外でReal-time PCRを用いた迅速診断法が開発されている[38]。

Ⅸ. わが国固有のヒストプラズマ症の遺伝子型

*H. capsulatum*の遺伝子多型はよく知られている。複数の遺伝子を組み合わせる多遺伝子解析やリボゾームRNA遺伝子のITS 1-5.8S-ITS 2領域の配列単独の解析によれば、*H. capsulatum* var. capsulatumと*H. capsulatum* var. *farciminosum*では旧来のvarietyとの整合性はなかったが、*H. capsulatum* var. *duboisii*は独自の遺伝子型が保たれていると報告されていた[24～26]。

近年、病原性真菌の系統関係、遺伝子多型、分子疫学的解析には、バーコード遺伝子といわれているリボゾームRNA遺伝子のITS 1-5.8S-ITS 2領域、約500～600塩基の配列を比較することによる解析が重要視されている[39]。

*H. capsulatum*もリボゾームRNA遺伝子のITS 1-5.8S-ITS 2領域配列を用いた樹系解析により、*H. capsulatum* var. *farciminosum*と*H. capsulatum* var. *capsulatum*由来配列の混在するグループ（分子疫