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Original Article

Histoplasma capsulatum variety *duboisii* Isolated in Japan from an HIV-infected Ugandan Patient

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Abstract

A strain of *Histoplasma capsulatum* var. *duboisii* (deposited as IFM 50954 in Chiba University) was isolated from the cerebrospinal fluid of a female Ugandan patient infected with HIV. The isolate had *in vitro* urease activity on Christensen's urea agar slants, although the common belief is that *H. capsulatum* var. *duboisii* is urease negative, and is, considered one of the characteristic markers that distinguishes the three varieties of *H. capsulatum*. Forty *H. capsulatum* var. *capsulatum*, five *H. capsulatum* var. *duboisii*, and five *H. capsulatum* var. *farciminosum* isolates were evaluated for urease activity on Christensen's urea agar slants and for other qualitative and quantitative urease assays of activity. All 50 isolates of *H. capsulatum* used in this study were positive for urease activity, suggesting that urease activity may be universal characteristic of *H. capsulatum*. We also compared the urease activity and pathogenicity of seven *H. capsulatum* isolates that convert into yeast-form cells. Although isolate IFM 50954 showed moderate virulence in mice and moderate urease activity among tested *H. capsulatum* isolates, there was no correlation between level of urease activity and pathogenicity. In addition, scanning electron microscopy revealed that some microconidia of isolate IFM 50954 formed "double-cell" configurations that were attached to each other by narrow bases.
key words: *Histoplasma capsulatum* var. *duboisii*, HIV, pathogenicity, urease

Introduction

Histoplasma capsulatum, the causative agent of histoplasmosis, is a thermo-dependent dimorphic fungus that takes a mycelial form at room temperature and transforms from the mycelial to the yeast form in host tissues or at 35-37℃ on certain culture media. Histoplasmosis occurs worldwide, especially in temperate, subtropical, and tropical regions. Histoplasmosis is classified into three types: histoplasmosis capsulati, which is caused by *H. capsulatum* var. *capsulatum*; histoplasmosis duboisii, which is caused by *H. capsulatum* var. *duboisii*; and histoplasmosis farciminosi, which is caused by *H. capsulatum* var. *farciminosum* (*H. farciminosum*)[1]. As of December 2002, 38 cases of histoplasmosis in humans had been reported in Japan: 34 cases of histoplasmosis capsulati, three of histoplasmosis duboisii, and one of histoplasmosis farciminosi[2]. Although histoplasmosis was once thought to be an imported fungal infection in Japan, several human, one equine and four canine cases were reported as autochthonous mycosis[2, 3].

Interestingly, the first autochthonous case of histoplasmosis duboisii in Japan was reported from Okayama Prefecture by Yamato *et al.* in 1957[4]. Although the case was described as histoplasmosis duboisii by Chandler *et al.*[5], the size of the yeast-like cells in the lung of the affected patient (10-15 μm in diameter), made it difficult to determine whether the disease really had been diagnosed correctly. After this case, two additional cases of histoplasmosis duboisii were reported[6, 7]. An isolate of *H. capsulatum* var. *duboisii* from an HIV-infected Ugandan female patient was deposited in our center-database as IFM 50954 for identification. The isolate was

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confirmed to be *H. capsulatum* var. *duboisii* by genotyping of the internal transcribed spacer (ITS) 1, 5.8S and ITS 2 region ribosomal RNA sequences[8].

According to de Hoog *et al*[9], *H. capsulatum* var. *duboisii* lacks urease activity, but other varieties are positive. Differentiation with these criteria was thought to be convenient as a means for preliminary identifiing the variety. We accidentally detected urease-positive phenotype of isolate IFM 50954, when IFM 50954 and IFM 50955 (*H. capsulatum* var. *capsulatum*) were tested simultaneously for urease activity on Christensen's urea agar slants (Eiken, Tokyo, Japan) at room temperature. Both isolates reacted as urease positive within 4 hours, although it was expected that IFM 50954 would be urease negative. Therefore, we attempted to determine the urease activities of the three varieties of *H. capsulatum*.

In the present study, we evaluated urease activities of 50 *H. capsulatum* isolates of three varieties stored at our center. The correlation between urease activity and virulence in mice was also examined for seven isolates that convert into yeast-form cells.

Materials and Methods

Isolates

Forty isolates of *H. capsulatum* var. *capsulatum* (IFM 5396, 5397, 5398, 5399, 5400, 5401, 5402, 5403, 5404, 5405, 5406, 5407, 5408, 5410, 5411, 5412, 5413, 5414, 40752, 41323, 41329, 41330, 41331, 41618, 41619, 46003, 46004, 46159, 47750, 49109, 49110, 49200, 49721, 50248, 50249, 50250, 50251, 50955, 50958, 50959), five isolates of *H. capsulatum* var. *duboisii* (5415, 5416, 5417, 41332, 50954), and five isolates of *H. capsulatum* var. *farciminosum* (5418, 41333, 41334, 41335, 41612) were used in this study.

Mycological studies

The mycelial form of isolate IFM 50954 was grown on slide cultures of potato dextrose agar (PDA, Difco, Detroit, MI, USA) at 25℃ for 4 weeks and observed by light microscopy after staining with lactophenol cotton blue.

Scanning electron microscopy (SEM) was done on isolate IFM 50954. Cells of the mycelial form of this isolate on a PDA slant grown at 25℃ for 2 months were pre-fixed with a drop of 40% formalin at room temperature for 24 hours. The formalin-treated cells were re-fixed with 3% glutaraldehyde in phosphate-buffered saline for 24 hours at 4℃. Fixed cells were post-fixed with 1% osmium tetroxide, dehydrated in ethanol (15-minute successive washes in 50% to 100% ethanol), and incubated twice for 15 minutes in iso-amyl acetate. Critical point-dried samples were coated with platinum-palladium before observation and collection of images with an S-800 scanning electron microscope (Hitachi, Tokyo) at 3 kV with magnifications between 3,000 and 15,000 x.

Mycelial-to-yeast form and yeast-to-mycelial form conversions were induced on brain heart infusion agar (BHI, Difco) supplemented with 1% dextrose slants at 35℃ for 1 week and on PDA slants at 25℃ for 1-2 weeks.

Control fungi for urease activity assays

A *Candida albicans* strain (IFM 40009) and a *Cryptococcus neoformans* strain (IFM 5844) were used in the qualitative assays as negative and positive controls, respectively.

Qualitative urease test on Christensen's urea agar

The mycelial forms of all 50 isolates of the three varieties of *H. capsulatum* were cultured at 25℃ on PDA for 7 days, each isolate was then one-point inoculated on Christensen's urea agar slants and cultured at 25℃. The onset and intensity of the color change were observed for 7 days and noted. The seven isolates that converted stably to yeast forms were tested similarly on Christensen's urea agar slants at 35℃.

Qualitative urease test by a colorimetric method in yeast-form cells

Seven isolates of *H. capsulatum* in yeast form were cultured at 35℃ on BHIA slants. The qualitative urease activity was measured with 2% urea (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as substrate. Briefly, 1 m*l* of fungal solution adjusted to 10^8 cells/m*l* was mixed with 1 m*l* of substrate solution supplemented with 0.1% phenol red as a color indicator and incubated at 35℃ for 24 hours. The subsequent color changes were recorded.

Quantitative urease assay by photometric measurement

Quantitative photometric measurement of urease activity was also performed by incubating cytosolic extracts of each of the 50 isolates with 2% urea solution. Fungal extracts from mycelial and yeast form cultures were obtained as follows: Each isolate was taken from 1-month-old PDA slant cultures and cultured for 2 weeks in 20 m*l* of potato dextrose broth (PDB, Difco) in 100-

ml flasks at 27°C with shaking at 120 rpm. Similarly, yeast-form cells were cultured at 35°C in 20 *ml* of brain heart infusion broth (BHIB, Difco) supplemented with 1% dextrose in 100 *ml* flasks for 1 week with shaking at 120 rpm. Both mycelial- and yeast- form cells were washed with sterilized water and centrifuged three times at 2,000 rpm for 5 minutes. Cells were homogenized manually with a glass homogenizer on ice. The fungal contents were centrifuged once at 3,000 rpm for 10 minutes at 4 °C and then passed through 0.45- μm filters (Millipore Corporation, Bedford, MA, USA).

The total protein content was determined by a modified Lowry method with a bicinchoninic acid kit for protein determination (Sigma-Aldrich, St. Louis, MO, USA) before the absorbance was measured at 562 nm (UV-1600, Shimadzu, Kyoto, Japan) in duplicate. A standard curve for the correlation coefficient between protein concentration and optical density (O.D.) at 562 nm was obtained from 5 concentrations (0.000, 0.125, 0.250, 0.500 and 1.000 mg/*ml* of albumin). The concentration of proteins from isolates was calculated with the formula $Y=1.181X+0.023$, with a confidence of $p<0.01$ and a range of 0 to 1.000 mg/*ml*.

Urease assay was performed by incubating 500 μl of each filtered fungal extract with 500 μl of 2% urea for 1 hour at 37°C on a shaker incubator. At the end of incubation, 500 μl of 1 N HCl was added to each reaction mixture to stop the reaction. After dilution of the mixture 60 times, absorbance at 450 nm was measured immediately and calculated against the total protein. Briefly, a mixture of 900 μl distilled water, 50 μl of incubated extract and 50 μl of Nessler's solution (Wako Pure Chemical Industries, Ltd.) was evaluated spectrophotometrically in duplicate. Urease activity was expressed in terms of amount of ammonia produced per mg of cellular protein per hour. The standard curve based on the correlation coefficient between the ammonia concentration and O.D. at 450 nm was obtained from 5 concentrations (0.000, 3.125, 6.250, 12.50 and 25.00 mM) of ammonia. The concentration of produced ammonia was then calculated from the formula $Y=0.004X+0.005$, with a confidence of $p<0.01$ and a range of 0 to 25.00 mM.

Pathogenicity

Virulence was evaluated with five isolates of *H. capsulatum* var. *capsulatum* and two isolates of *H. capsulatum* var. *duboisii*; these isolates constantly converted to the yeast form at 35°C on BHIA slants. Seven-day-old cultures were used.

Animals

Thirty-five 6-week-old male ddY mice (Nihon SLC, Shizuoka, Japan) were used. They were divided into 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 10^6 yeast cells/0.2 *ml* in sterilized normal saline *via* the caudal vein. Morbidity and mortality profiles were observed for 4 weeks. Surviving mice were killed with deep ether anesthesia. This work complied with all relevant guidelines and policies of the Animal Welfare Committee of the Faculty of Medicine of Chiba University, Japan.

Organ culture

Seven organs (brain, heart, lung, kidney, liver, spleen and the bone marrow from the femoral bones) of dead or sacrificed animals were examined macroscopically. With the exception of bone marrow, organs were cut into approximately 5 mm^3, placed on PDA plates, and cultured at 25°C for 2 weeks. After cutting the caput and caudal parts of the femoral bone, the bone marrow was pushed out with an 18 gauge needle and placed directly on a PDA plate. Fungal sprouts from each organ were recorded, and one point was given for each fungus-positive organ. These data are presented as the ratio of the number of positive organs to the number of animals tested for each isolate. The pathogenicity score was then expressed as the mean number of positive organs per mouse ± SD (standard deviation). Therefore, the maximal point value was seven, which means fungal sprouts were observed in all seven organs. The mean value from each isolate was plotted to assess the correlation between urease activity and pathogenicity[10].

Histopathological observation

Six organs (brain, heart, lung, kidney, liver, and spleen) obtained from dead or killed animals were examined histopathologically. Organs were fixed with buffered 10% formalin and processed by routine histopathological methods. The paraffin-embedded specimens were cut into 4 μm thick sections and stained with hematoxylineosin (HE) and by the periodic acid Schiff (PAS) reaction. Examination under a light microscope was done to determine the extent of fungal invasion of the organs. When fungal elements were observed in sections from an organ, the organ was assigned one point. These data were recorded as the ratio

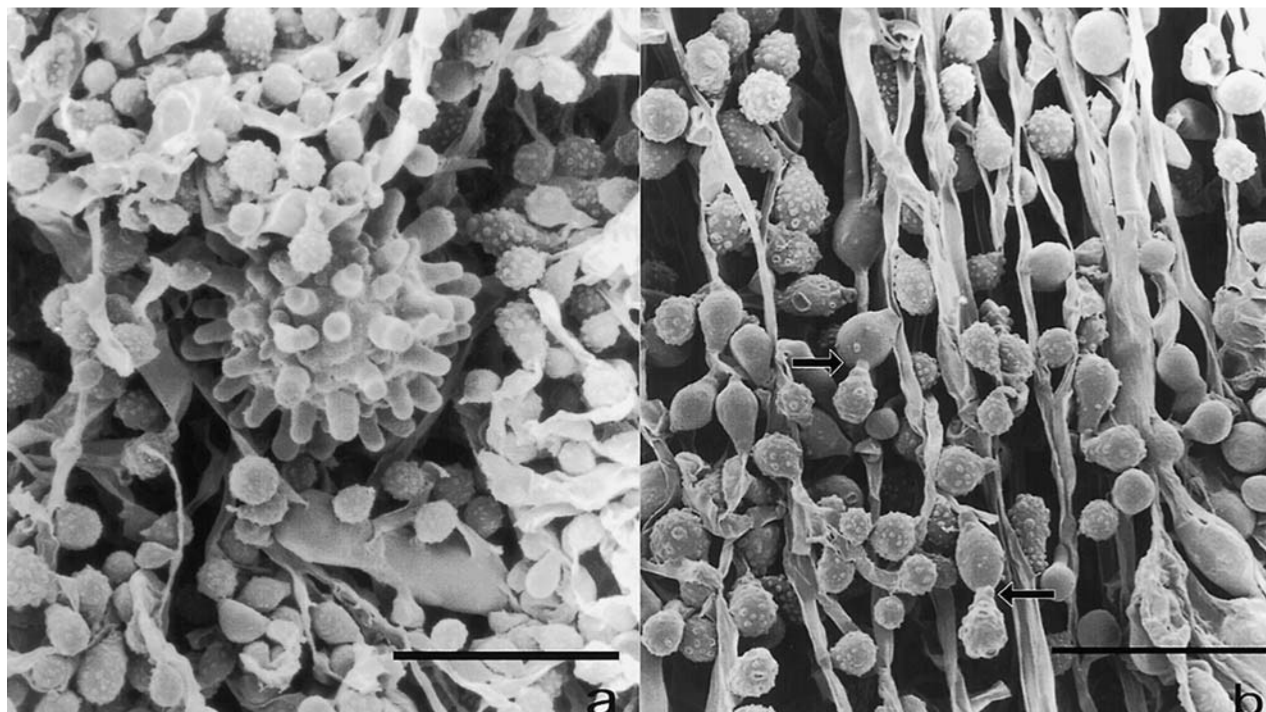

Fig. 1. Scanning electron microscopic images of *H. capsulatum* var. *duboisii* (IFM 50954).

(a) A large macroconidia is visible at the center and the surface exhibits multiple digitate protuberances (bar 10 μm).
(b) "Double-cell" configurations of microconidia are indicated by arrows (bar 10 μm).

of the number of organs in which yeast cells were observed to the number of animals tested. Score of the pathogenicity for histopathological observation was expressed as the mean number of positive organs per mouse ± SD. The maximun number of points was six indicating that fungal elements were observed in all six organs. The mean value for each isolate was plotted to assess the correlation between urease activity and pathogenicity[10].

Statistical analysis

Quantitative urease activity was analyzed by Student's *t*-test. The outstanding value from the quantitative urease activity assay among varieties was analyzed by Smirnov's rejection method. A *p*-value less than 0.05 was considered significant. The correlation between quantitative urease activity and virulence scores from organ culture and histopathological observations in mice were evaluated with the method of least squares[10]. The above analyses were performed with Instat, ver. 2.03 for Mac software (Informatique, Inc., Akasaka Tokyo).

Results

Isolate IFM 50954 produced both abundant macroconidia and microconidia on PDA slant cultures at 25°C after 4 weeks of culture (Fig 1a). The surfaces of the macroconidia appeared to have multiple digitate protrusions. Some microconidia were present in pairs, joined by a narrow base as observed by SEM (Fig 1b).

Seven of the fifty isolates used in this study converted stably to their yeast forms on BHIA slants at 35°C in 1 week. Five belonged to *H. capsulatum* var. *capsulatum* (IFM 5414, IFM 40752, IFM 41329, IFM 49721 and IFM 50249) and two belonged to *H. capsulatum* var. *duboisii* (IFM 5415 and IFM 50954). Yeast-form cells of the five *H. capsulatum* var. *capsulatum* isolates had diameters ranging from 1〜10 μm, and, those of the two *H. capsulatum* var. *duboisii* isolates ranged from 1〜15 μm in diameter.

The urease activity on Christensen's urea slants was positive for all fifty isolates within 7 days at 25°C. All isolates began to change color from a yellow to a slight pinkish by 4 hours of incubation. Christensen's urea agar slants inoculated with *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *farciminosum* isolates showed intense color changes to pinkish-red after 24 hours of incubation, whereas those with *H. capsulatum* var. *duboisii* isolates changed to pinkish-yellow with the exception of isolate IFM 50954, which changed to pinkish-red. Slants inoculated with yeast-form cells of *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii* isolates turned pinkish-red within 4 hours.

Qualitative measurements of urease activities of

Table 1. Urease activities of three varieties of *Histoplasma capsulatum*

Fungal species or variety	Number of isolates	Urease activity		
		Christensen urea agar in 4 hours	Qualitative colorimetric test in 7 days	Oualitative photometric urease assay* (mean ± SD)
Histoplasma capsulatum				
var. *capsulatum*	40	all+	all+	285.10 ± 133.97
var. *duboisii*	5	all+	all+	170.15 ± 184.21
var. *farciminosum*	5	all+	all+	507.11 ± 386.82
Candida albicans	1	−	−	ND
Cryptococcus neoformans	1	+	+	ND

*, data indicated as units/mg protein/h. No significant difference among quantitative photometric urease assay.
ND, not done; SD, standard deviation.

Table 2. Quantitative photometric urease assay of mycelial and yeast forms of *Histoplasma capsulatum* isolates

Variety IFM no.	Urease activity in mycelial form (units/mg protein/h)	Urease activity in yeast form (units/mg protein/h)
Histoplasma capsulatum var. *capsulatum*		
5414	211.76	625.94
40752	215.89	1002.91
41329	464.99	238.82
49721	177.79	759.21
50249	119.68	424.15
Histoplasma capsulatum var. *duboisii*		
5415	175.40	137.90
50954	486.60	525.02

Table 3. Quantitative photometric urease assay of *Histoplasma capsulatum* var. *duboisii*

IFM no.	Units/mg protein/h
5415	175.40
5416	75.66
5417	36.34
41332	76.73
50954	486.60*

* rejected by the Thompson-Smirnov's rejection analysis criteria, $p<0.05$.

yeast-form cells yielded positive results within 10-30 minutes. With the exception of the medium inoculated with isolate IFM 5415, which remained light pink after 4 hours of incubation, all isolates converted to a deep pink. The medium became a pinkish-red after 24 hours in all yeast-form isolates including IFM 5415.

Urease activities of mycelial-form cells were highest in *H. capsulatum* var. *farciminosum* followed by *H. capsulatum* var. *capsulatum* and then *H. capsulatum* var. *duboisii*. However, the difference in urease activiy was not statistically significant among the varieties by Student's *t*-test (Table 1). Yeast-form cells also contained urease activity (Table 2). Mycelial-form cells of isolate IFM 50954 showed comparatively higher levels of urease activity among the variety which was rejected by the Thompson-Smirnov's rejection analysis criteria ($p<0.05$, Table 3).

All five mice inoculated with IFM 5414 died between days 10 and 15 after inoculation; two mice died on day 10, one on day 11, one on day 12, and the last mouse of this group died on day 15. Mice infected with the other four isolates belonging to *H. capsulatum* var. *capsulatum* and two isolates belonging to *H. capsulatum* var. *duboisii* did not die. These mice were killed for organ culture and histopathological examination on day 28 after inoculation.

Fungal cells were recovered from all organs of mice inoculated with in isolate IFM 5414 (*H. capsulatum* var. *capsulatum*) indicating that this isolate has the highest virulence. The virulence score for organ culture of isolate IFM 50954 was in the middle, whereas that of isolate IFM 5415 was the lowest. The remaining *H. capsulatum* var. *capsulatum* isolates showed various scores (Table 4).

Mice infected with isolate IFM 5414 showed severe damage to all organs except for brain, histopathologically. Abundant fungal organisms 1 ~ 8 μm in diameter were detected inside liver cells and a few migrating macrophages (Fig 2a). Isolate IFM 50954 (*H. capsulatum* var.

Table 4. Organ-culture scores of mice infected with the seven *Histoplasma capsulatum* isolates

Isolate tested	Number of animals	Organs with positive-cultures/Total organs cultured							Mean score±SD
		Liver	Kidney	Spleen	Lung	Heart	Brain	Bonemarrow	
Histoplasma capsulatum var. *capsulaatum*									
IFM 5414	5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	7.00±0.000
IFM 40752	5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	0.60±0.548
IFM 41329	5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0.00±0.000
IFM 49721	5	5/5	5/5	5/5	5/5	5/5	4/5	5/5	6.80±0.447
IFM 50249	5	5/5	3/5	5/5	4/5	5/5	4/5	5/5	6.20±1.300
Histoplasma capsulatum var. *duboisii*									
IFM 5415	5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0.00±0.000
IFM 50954	5	2/5	0/5	3/5	0/5	0/5	0/5	0/5	1.00±0.707

SD, standard deviation.

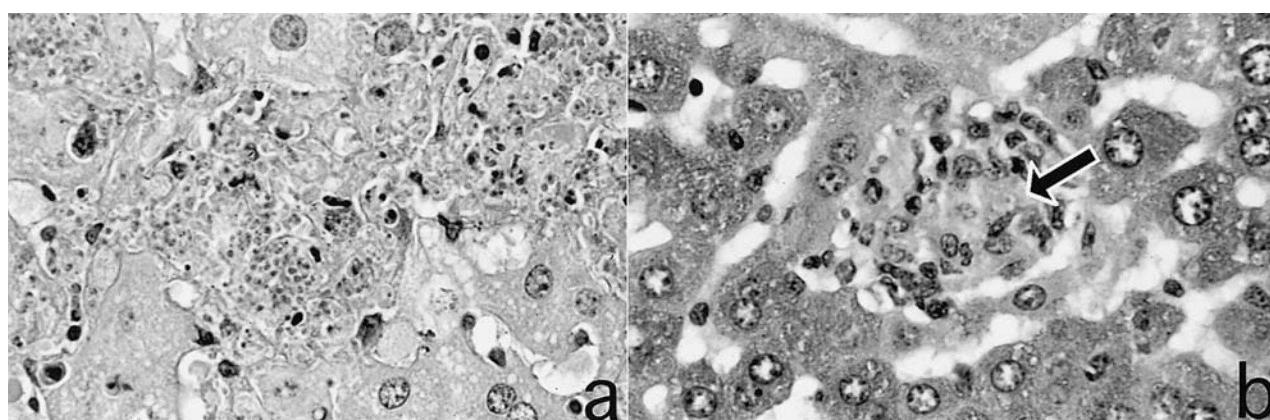

Fig. 2. Histopathological observations of the livers from *H. capsulatum*-inoculated mice.

(a) Intracellular localization without host reaction in a mouse infected with *H. capsulatum* var. *capsulatum* isolate IFM 5414 (PAS, ×400).

(b) A few yeast cells surrounded by granulomatous cells in a mouse infected with *H. capsulatum* var. *duboisii* isolate IFM 50954 (PAS, ×400).

Table 5. Scores of histopathological findings taken from the six internal organs of mice infected with the seven *Histoplasma capsulatum* isolates

Isolate tested	Number of animals	No. of organs where fungal presence was observed						Mean score±SD
		Liver	Kidney	Spleen	Lung	Heart	Brain	
Histoplasma capsulatum var. *capsulatum*								
IFM 5414	5	5/5	5/5	5/5	5/5	5/5	1/5	5.20±0.447
IFM 40752	5	3/5	1/5	5/5	1/5	2/5	0/5	2.40±1.517
IFM 41329	5	4/5	2/5	5/5	1/5	0/5	0/5	2.40±1.140
IFM 49721	5	4/5	3/5	5/5	2/5	2/5	0/5	3.20±1.304
IFM 50249	5	5/5	1/5	5/5	5/5	4/5	2/5	4.40±1.140
Histoplasma capsulatum var. *duboisii*								
IFM 5415	5	0/5	0/5	5/5	1/5	0/5	0/5	1.20±0.447
IFM 50954	5	4/5	0/5	5/5	1/5	3/5	0/5	2.60±0.548

SD, standard deviation.

duboisii) also showed intracellular localization of yeast-form cells (7～15 μm) in the liver and was surrounded by granulomatous cells (Fig. 2b). Isolate IFM 50954 caused moderate organ damage, whereas isolate IFM 5415 showed the least. The organs of mice infected with remaining isolates showed granulomatous lesions to variable degrees. In general, the brain appeared to be the organ with the lowest level of invasion and damage (Table 5). There was no correlation between urease activity and scores of organ cultures or histopathological observations.

Discussion

In the present study we found that three varieties of *H. capsulatum* have urease activity. Isolate IFM 50954 was urease-positive by quantitative urease assay and showed strong urease activities both for the mycelial and yeast forms. Our findings contradict previous reports that *H. capsulatum* var. *duboisii* lacks urease activity, whereas other varieties are positive[9]. Although it was thought that this criterion is useful for differentiation between varieties, even in urease activity assay on Christensen's urea agar slants judged within a few hours after inoculation, differentiation of the varieties was not possible, because of the exception isolate of *H. capsulatum* var. *duboisii*, IFM 50954. In fact, through measurement by quantitative and qualitative assays we found that all varieties of *H. capsulatum* are urease positive. The characteristic for differentiation of the three varieties of *H. capsulatum* thus should be reconsidered.

The present study started as the result of an encounter with *H. capsulatum* var. *duboisii* isolated from an HIV-infected Ugandan patient. This isolate had urease activity as strong as that of *H. capsulatum* var. *capsulatum*. The isolation of fungi with atypical characteristics from HIV-infected patients undergoing anti-retrovirus treatment has been reported[11-13]. These fungi occasionally present with atypical morphology or unusual biochemical characteristics. Isolate IFM 50954 was also isolated from an HIV-infected patient who had undergone anti-retrovirus therapy. It showed significant urease activity *in vitro* among the varieties with characteristics of "double cell" formation in microconidia, which has not been reported in *H. capsulatum* isolates. However, this unusual arrangement of microconidia in this isolate should be confirmed after observation of many *H. capsulatum* strains.

The urease activity has been suggested to be a marker of virulence for many bacteria, including *Helicobacter pylori* and *Proteus mirabilis*[14, 15]. These organisms may cause toxicity through alkaline degradation of various host proteins, or urease may aid in promoting survival of pathogens by rendering a host defense. Many fungi are known to have urease activity, and, many other virulence factors such as capsular formation, laccases and phenoloxidases in addition to urease activity, have also been reported[16]. The role of urease as an obvious virulence factor has been recognized in *Cryptococcus neoformans*[17]. Although we anticipated finding a correlation between urease activity and pathogenicity in the seven *H. capsulatum* isolates, our data did not indicate such a correlation. Therefore, other virulence factors should be considered.

There are some side effects of anti-retrovirus treatment for pathogenic fungi[13]. This could be due to unknown effects of the virus or drug on immune suppression caused by the virus. Some patients with HIV are profoundly anemic, and carbon dioxide (CO_2) has been implicated as an activator of the urease apoenzyme[18]. Even if constitutive urease production by an organism is low, the low oxygen tension in conjunction with higher CO_2 tension usually observed under anemic conditions, may trigger urease production *in vivo*. Isolate 50954 might acquire relatively strong urease activity through the above process.

In conclusion, three varieties of *H. capsulatum* have universal urease activity. Molecular biological differentiation is the best method to distinguish the 3 varieties of *H. capsulatum*, at present.

Acknowledgments

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