underlying respiratory diseases such as tuberculosis sequelae, chronic obstructive pulmonary disease, or may be associated with pulmonary surgery, radiotherapy, pneumococcal infection, diabetes mellitus, collagen disease, corticosteroid-induced immunosuppressive conditions. In most cases, CPA requires long-term treatment [7,8,13], but there is limited information available on CPA management.

Micafungin (MCFG), an echinocandin antifungal agent, is a selective inhibitor of the synthesis of (1,3)-beta-D-glucan, a primary component of fungal cell walls. MCFG exerts its fungicidal action on *Candida*, and causes rupture of mycelial tips of *Aspergillus* by its potent inhibition of mycelial extension [14–17]. Although various clinical studies have demonstrated the effectiveness of MCFG for invasive fungal infections [18–29], there are few reports on its use for the treatment of CPA [25,30]. We recently published the first large scale prospective study comparing the efficacies of intravenous MCFG and intravenous voriconazole in the treatment of CPA [31]. There was a favorable response rate with both MCFG (60.0%) and voriconazole (53.2%) but fewer side-effects were reported for MCFG (26.4%) than for voriconazole (61.1%) [31]. We have now conducted another prospective observational study to clarify the efficacy and safety profile of MCFG with 38 CPA patients admitted to 28 Japanese medical institutions between April 2003 and March 2005. While this present study is relatively small, non-randomized observational investigation, the information obtained from a clinical setting could also be of a benefit to clinicians dealing with CPA.

Materials and methods

Study patients

The enrollment criterion for prospective clinical studies of CPA proposed by Denning *et al.* [7] was slightly modified, and utilized in this investigation. Briefly, patients were enrolled if they had clinical symptoms caused by pulmonary aspergillosis (any of cough, sputum, hemosputum, hemoptysis, and pyrexia (≥ 37.0°C axillary temperature), and elevated levels of inflammatory markers (C-reactive protein value, white blood cell count, and erythrocyte sedimentation) before the treatment with MCFG. In addition patients had to meet at least one of the following three criteria:

1. The causative agent identified as *Aspergillus* spp. by recovery in culture or histopathological examination;
2. The appearance of new nodules or the expansion of the existing nodules on a chest X-ray or computed tomography (CT) which are suspected to be aspergillosis and positive serological or mycological tests results; and/or
3. Suspected complex aspergilloma with positive feature on a chest X-ray or CT.

Patients with features of invasive pulmonary aspergillosis (i.e., symptoms of less than 1 month) and allergic bronchopulmonary aspergillosis were excluded.

Patients were treated with MCFG for 4–84 days with doses of 50–150 mg once daily by intravenous drip infusion. In severe or refractory cases, an increase in dosage up to 300 mg daily was allowed. Since no data are available about the dose effect of MCFG, changes in dosage were determined by attending physicians based on the severity of disease.

The following information was reviewed from the patient's medical records; patient characteristics (e.g., sex, age, body weight), treatment conditions (e.g., dose of MCFG, duration of MCFG therapy, all other antifungals or antibiotics used during the seven days prior to the initiation of MCFG therapy, other concomitant therapies), clinical symptoms or findings, radiological findings obtained through chest X-ray or CT, and adverse events during therapy.

This study was approved by the Institutional Review Board at each institution. Since this was an observational study, informed consent was not required.

Efficacy assessment

The efficacy of MCFG was based on improvements in CPA-related clinical symptoms and radiological findings such as chest X-ray and CT. Improvement in clinical symptoms were rated as 'improved', 'worsened' or 'unchanged'. Changes in the radiological findings from chest X-ray and CT were rated as 'improved' when the shadows on the images were reduced or diminished, 'worsened' when the shadows were increased, and 'unchanged'.

Overall clinical efficacy ('effective' or 'not effective') was determined on the basis of both clinical symptoms and radiological findings. Overall clinical efficacy was assessed as 'effective' when clinical symptoms were rated as 'improved' and radiological findings were not rated as 'worsened'. Overall clinical efficacy was also assessed as 'effective' when radiological findings were rated as 'improved' and the clinical symptom was not rated as 'worsened'. Overall clinical efficacy was assessed as 'not effective' when both the clinical symptoms and the radiological findings were rated as 'unchanged' or either of them was rated as 'worsened'.

Safety assessment

All adverse events, including abnormal laboratory findings noted after the initiation of MCFG therapy, were recorded.

Adverse events that the investigator suspected to have a causal relationship with MCFG were classified as adverse drug reactions, and the seriousness was classified into three levels of 'mild', 'moderate (neither mild nor serious)', and 'serious' in accordance with the ICH Harmonised Tripartite Guideline [32].

Statistical analysis

The data were expressed as mean ± SD. Categoric variables were expressed as a percentage and were analyzed by the Fisher exact test. A P-value of < 0.05 was considered statistically significant.

Results

Patient characteristics

Table 1 summarizes patient characteristics. The patients consisted of 25 males (65.8%) and 13 females (34.2%) and were predominately 65 years or older (27 patients [71.1%], maximum; 90 years, mean: 68.8 years) with a mean body weight of 45.1 kg. The duration of MCFG therapy was 14 days or less in 6 patients (15.8%), 15-28 days in 13 patients (34.2%), and 29 days or longer (up to a maximum of 84 days) in the remaining 19 patients (50.0%). The mean duration was 33.7 ± 19.9 days, the mean daily dose was 167.0 ± 54.4 mg/day, with approximately half of the patients (55.3%) receiving 150 mg/day.

Table 1 Patient characteristics.

Characteristic	Number of patients
Total	38
Sex	
Male	25
Female	13
Age (years)	
23–64	11
65–90	27
(Mean ± SD)	(68.8 ± 10.9)
Body weight (kg)	
(Mean ± SD)	(45.1 ± 10.6)
Duration of treatment (days)	
4–14	6
15–28	13
29–84	19
(Mean ± SD)	(33.7 ± 19.9)
Mean daily dose (mg/day)	
100	3
> 100 to < 150	5
150	21
> 150 to < 300	5
300	4
(Mean ± SD)	(167.0 ± 54.4)

Mean ± SD, Mean ± Standard Deviation.

Table 2 Clinical response by clinical symptoms and radiological findings.

Clinical response (%)	Clinical symptoms	Radiological findings
Effective 26/38 (68.4%)	Improved (23)*	Improved (10)* Unchanged (13)*
	Unchanged (3)*	Improved (3)*
Non effective 12/38 (31.6%)	Unchanged (6)*	Unchanged (4)* Worsened (2)*
	Worsened (6)*	Unchanged (4)* Worsened (2)*

*Number of patients.

Clinical efficacy

Clinical response by clinical symptoms and radiological findings (Table 2). Overall clinical efficacy was assessed as 'effective' in 26 patients (overall clinical efficacy rate: 68.4%), of whom 10 showed improvement in both clinical symptoms and radiological findings, and improvements in either clinical symptoms or radiological findings was noted with the remaining 16 patients. Of the 12 patients in which overall clinical efficacy was assessed as 'not effective', two patients showed worsening in both clinical symptoms and radiological findings, and 10 patients had either worsening clinical symptoms or radiological findings.

Clinical response by duration of treatment and mean daily dose (Table 3). The observed clinical efficacy rate when analyzed by duration of treatment was lower in patients treated for 14 days or less (3/6 patients) than in the other groups. The efficacy rates were 92.3% in patients treated for 15–28 days, and 57.9% in patients treated for 29–84 days. The clinical efficacy rate of patients treated with: (1) 100 mg/day, (2) more than 100 mg/day and less than 150 mg/day, (3) 150 mg/day, (4) more than 150 mg/day and less than 300 mg/day, and (5) 300 mg/day were 100.0% (3/3 patients), 80.0% (4/5 patients), 66.7% (14/21 patients), 60.0% (3/5 patients), and 50.0% (2/4 patients), respectively.

Table 3 Clinical efficacy rates by duration of treatment with micafungin and mean daily dose.

Variable	Clinical efficacy rate (%)		Statistical test*
Total	26/38	(68.4)	–
Duration of treatment (days)			
4–14	3/6	(50.0)	$P = 0.050$
15–28	12/13	(92.3)	
29–84	11/19	(57.9)	
Mean daily dose (mg)			
100	3/3	(100.0)	$P = 0.786$
> 100 to < 150	4/5	(80.0)	
150	14/21	(66.7)	
> 150 to < 300	3/5	(60.0)	
300	2/4	(50.0)	

*Fisher exact test.

Safety

Incidence of adverse drug reactions (Table 4). A total of 38 adverse events occurred in 16 of 38 patients of which 10 events in six patients (15.8%) were regarded as adverse drug reactions related to MCFG. Common adverse drug reactions were reported as seven events of abnormal liver functions including increased aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (γ-GT), and blood alkaline phosphatase (ALP). Among the 10 adverse drug reactions, the seriousness was classified as moderate for five reactions and mild for five reactions. No serious adverse drug reactions classified by the ICH guideline [32] were observed. Treatment with MCFG was discontinued in one patient because of increases of AST from 40–199 U/L and ALT from 36–175 U/L. The investigator assessed these events as moderate, and suggested the contribution of concomitant drugs such as arbekacin and imipenem/cilastatin.

Incidence of adverse drug reactions by age, dose, and duration (Table 5). The incidence of adverse drug reactions by age was 9.1% (1/11 patients) in those under 65 years and 18.5% (5/27 patients) in those 65 years or older. The latter age group included six patients aged 80 years or older, in whom the incidence of adverse drug reactions was 16.7% (1/6 patients). The incidence of adverse drug reactions in patients treated with 150 mg/day was 19.0% (4/21 patients). The remaining two patients with whom adverse drug reactions were observed, were treated with 117 mg/day and 300 mg/day, respectively. The incidence of adverse drug reactions by duration of treatment was 33.3% (2/6 patients) in patients treated for 14 days or less, 15.4% (2/13 patients) in patients treated for 15-28 days, and 10.5% (2/19 patients) in patients treated for 29–84 days.

Discussion

A multicenter, observational study of 38 CPA patients treated with MCFG showed that the overall clinical efficacy rate of the antifungal was 68.4% (26/38 patients), which is comparable to the results of our previous studies [25,31] despite the fact that the design of investigations were different. CPA in which mycelia do not penetrate the surrounding cavity wall is generally defined as simple aspergilloma, but in practice it is often difficult to clearly distinguish between CNPA, CCPA, and CFPA. Indeed, Hope *et al.* consider these pathological conditions to be a continuous series of the infection process [13]. Therefore, we considered the enrollment criteria proposed by Denning *et al.* [7] the most practical and utilized it as inclusion criteria in this study.

The clinical trial of MCFG conducted for marketing approval also demonstrated clinical efficacy rates of 67% (6/9 patients) for CNPA, and 55% (12/22 patients) for aspergilloma [25]. The efficacy of MCFG shown in this study with patients of various backgrounds in a post-marketing setting seems comparable with that of the above-mentioned trial, although there are still differences in methodologies, baseline patient characteristics, dosage regimens, and timing of assessments.

The Infectious Diseases Society of America guidelines for aspergillosis recommend MCFG as an alternative therapy to voriconazole for CNPA and CCPA. For simple aspergilloma, the guidelines propose surgical resection as the primary treatment, and oral itraconazole and voriconazole as alternative therapies in those cases where surgery was not possible [8]. In our studies MCFG achieved an overall clinical efficacy rate of approximately 60–70%. This result and those of comparison studies with MCFG and voriconazole suggest that MCFG has potential as an alternative therapy to itraconazole and/or voriconazole.

There are no significant differences between the efficacy and the dose of MCFG. However, the clinical efficacy

Table 4 Profile of adverse drug reactions (by seriousness).

Type of adverse drug reaction*	Number of events	Seriousness		
		Serious	Moderate	Mild
Abnormal liver function (including increased AST, ALT, gamma-GT, and ALP)	7	0	3	4
Injection site extravasation	1	0	1	0
Edema peripheral	1	0	1	0
Eosinophil count increased	1	0	0	1
Total	10	0	5	5

*Determined by investigator to be definitely, probably, or possibly drug related. AST, aspartate aminotransferase; ALT, alanine aminotransferase; gamma-GT, gamma-glutamyltransferase; ALP, alkaline phosphates.

Table 5 Incidence of adverse drug reactions by age, mean daily dose or Duration of treatment (days).

Variable	Incidence ofadverse drug reactions (%)	Statistical test*
Total	15.8 (6/38)	–
Age (years)		
23–64	9.1 (1/11)	$P = 0.650$
65–90	18.5 (5/27)	
Mean daily dose (mg/day)		
100	0.0 (0/3)	$P = 0.898$
> 100 to < 150	20.0 (1/5)	
150	19.0 (4/21)	
> 150 to < 300	0.0 (0/5)	
300	25.0 (1/4)	
Duration of treatment (days)		
4–14	33.3 (2/6)	$P = 0.410$
15–28	15.4 (2/13)	
29–84	10.5 (2/19)	

*Fisher exact test.

rate in patients who were treated with more than 150 mg was relatively lower than other patients. Nine of the patients who were treated with more than 150 mg/day included five responders and four non-responders. No obvious differences were found in clinical symptoms/inflammatory findings at the start of MCFG treatment between these two groups of patients. On the other hand, a comparison of the radiological findings revealed that only 1 of 5 responders was found to have severe disease as indicated by shadows covering 2/3 or more of either lung field, whereas 3 of 4 non-responders were considered to have severe disease with shadows covering almost the entire unilateral lung fields. In order to clarify the relationship between the severity of the disease and the MCFG dose, an additional clinical study in a larger patient population will be needed in the future.

It is difficult to draw a clear conclusion on the appropriate duration of treatment of MCFG from this observational study, in which each investigator determined whether MCFG therapy should be continued or discontinued. However, the patients treated with MCFG for 15–28 days and those treated for 29 days or longer showed slightly higher efficacy rates in comparison to those in which therapy was discontinued within 14 days. These data may suggest that treatment for more than 14 days is recommended, although the number of patients treated for up to 14 days was limited in this study. Therefore, the appropriate duration of treatment based on each patient's condition should further be explored in the future.

With regard to the safety of MCFG, the incidence of adverse drug reactions was 15.8% (6/38 patients), and abnormal liver functions were the most common reported drug reaction. In the Japanese open-label, non-controlled clinical trial of MCFG for marketing approval of 70 deep-seated mycosis patients, 33 adverse drug reactions occurred in 21 patients (incidence: 30.0%) [25]. Our MCFG and voriconazole comparative study also indicated incidence rate of 26.4%, which is not different from a previous study [31]. Common reactions included increases in hepatic enzymes like γ-GT and blood ALP, blood urea nitrogen, and creatinine. None of these reactions were assessed as serious [25,31]. Even though the present study was conducted in CPA patients with varying backgrounds, no marked differences were found between these three studies concerning the type or seriousness of the adverse drug reactions.

When the incidence of adverse drug reactions was analyzed in terms of contributing factors, no relationship was observed between the incidences and increase in age, dose or duration of treatment. In addition, we were able to assess adverse drug reactions in patients not enrolled in the clinical trial of MCFG for marketing approval, particularly patients aged 80 years or older, in patients treated at a dose of more than 150 mg/day and treated for 57 days and longer. The only adverse drug reaction reported in the patient aged 80 years or older was an abnormal liver function, which did not result in discontinuation of the treatment and was reported to have subsided 24 days after onset. The patient treated at a dose of more than 150 mg/day experienced three events of abnormal liver functions, of which two were classified as moderate and one as mild. Concomitant drugs such as arbekacin and imipenem/cilastatin may also have contributed to the onset of these adverse drug reactions, as well as MCFG, in this patient. The outcomes of these adverse drug reactions were not traceable in this case because of death caused by aggravation of the underlying disease. No adverse drug reaction was observed after treatment with MCFG for 57 days and longer.

In conclusion, MCFG achieved satisfactory treatment results in CPA patients with varying backgrounds. The safety profiles of MCFG obtained from this study was similar with other previous studies. Accumulation of clinical data will be beneficial in the management of CPA patients.

Declaration of interest: All authors have received consultation fees from Astellas Pharma Inc. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 3 March 2011.

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Medical Mycology Month 2011, Early Online, 1–6

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Case Report

A case of bronchial aspergillosis caused by *Aspergillus udagawae* and its mycological features

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Aspergillus udagawae and *A. fumigatus* share similar morphological features but they differ genetically. There is also an important clinical distinction as *A. udagawae* is less sensitive to amphotericin B than *A. fumigatus*. We encountered a rare case of bronchial infection due to *A. udagawae* that was successfully treated with voriconazole. An 82-year-old woman with diabetes mellitus complained of bloody sputum. Bronchoscopy revealed a white plugged region at the origin of the right bronchi B5. Cytological study revealed a clot composed of filamentous fungi and *Aspergillus* spp. was detected by culture. Molecular analysis revealed that the causative agent was *A. udagawae*, and voriconazole was used for the treatment. In comparison to *A. fumigatus*, the *A. udagawae* strain isolated in this case was less sensitive to amphotericin B, less virulent in immunosuppressed mice, and more sensitive to hydrogen peroxide, features that are almost identical to those of the previously reported isolates of the fungus. We should be aware of the emergence of new *Aspergillus* species that might pose a clinical threat.

Keywords *Aspergillus udagawae*, diabetes mellitus, bronchial aspergillosis

Introduction

Aspergillus udagawae belongs to the *Aspergillus* section *Fumigati* and is quite similar morphologically to *A. fumigatus*. Recent studies have shown that *A. udagawae* is genetically dissimilar to *A. fumigatus*, and that *A. udagawae* is less sensitive to amphotericin B [1]. Only a few cases of its involvement in human infections have been reported to date [2–4]. Here, we provide the first description of a case of bronchial aspergillosis caused by *A. udagawae* in a patient with mild diabetes mellitus. Since there is only limited information available we conducted studies of the mycological features of the clinical isolates of this fungus [5], *in vitro* and *in vivo* experiments regarding its drug susceptibility, pathogenicity in mice, and sensitivity to hydrogen peroxide.

Case report

An 82-year-old female patient with diabetes mellitus visited Goto Central Hospital complaining of bloody sputum of two days duration. Chest X-ray revealed infiltrates in the right middle lung field and computed tomography (CT) image showed segmental infiltrates in the right middle lobe (Fig. 1A). She was immediately admitted for further examination and treatment. On admission, her vital signs were as follows: height, 145 cm; body weight, 51 kg; body temperature, 37.1°C; heart rate, 76 beats/min with a regular rhythm; blood pressure, 130/64 mmHg; respiratory rate,

Received 5 August 2011; Received in final revised form 28 October 2011; Accepted 1 November 2011

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DOI: 10.3109/13693786.2011.639036

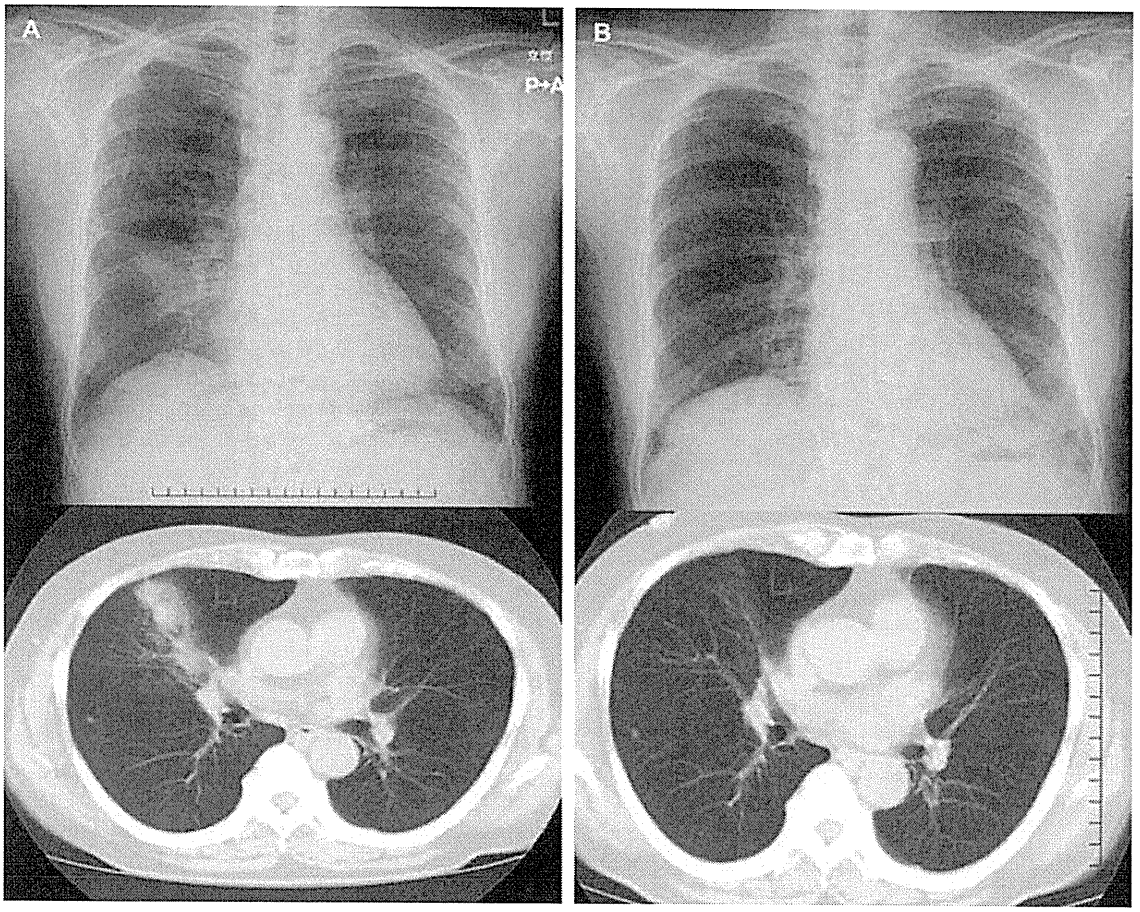

Fig. 1 Imaging findings upon admission and after antifungal drug administration. (A) Chest X-ray shows infiltrates in the right middle lung field and computed tomography image shows segmental infiltrates at the right middle lobe. (B) Chest X-rays show absence of infiltrates after 4 months of treatment with antifungal agent.

12 breaths/min; and SpO_2, 97% (room air). Physical examination revealed no rales, murmurs, or signs of systemic lymphadenopathy, hepatosplenomegaly, pretibial edema, and neurological abnormalities. Laboratory findings upon admission were as follows: white blood cell count, 12280/μl with a shift to the left (neutrophils, 80%); C-reactive protein, 0.26 mg/dl; erythrocyte sedimentation rate, 19 mm/h; β-D glucan, 5.4 pg/ml (cut-off, 20 pg/ml); and HbA1c, 6.8%. Test for precipitating antibodies to *Aspergillus* was negative, serum *Aspergillus* galactomannan antigen (ELISA) test results were positive at 1.4 (cut-off, 0.5) and IgE (RAST) test results against *Aspergillus* were negative. Bacterial pneumonia was suspected, and therefore, therapy with sulbactam/ampicillin (4.5 g/day) was initiated. Two days after admission, bronchoscopy revealed a white plug-like polypoid region with a hemorrhagic tendency at the origin of the right bronchi B5 (Fig. 2). A biopsy from this region was avoided because of the hemorrhagic tendency, and as a result, brushing followed by washing with saline was subsequently performed. Cytological examination of the bronchial lavage fluid revealed a clot composed of Y-shaped filamentous fungi with septa and microbiological studies detected *Aspergillus* spp. No other microorganisms including bacteria were isolated.

Bronchial aspergillosis was diagnosed and the therapy with antibacterial drugs was changed to 400 mg/day of voriconazole (VRCZ). The bloody sputum gradually resolved and disappeared after six days of VRCZ treatment. Chest X-ray showed improvement eight days after VRCZ administration. The patient was discharged on day 24 and oral VRCZ was continued on an outpatient basis. Two weeks after discharge, liver dysfunction was identified by an elevated aspartate aminotransferase, 53 IU/L (normal range: 8–38 IU/L), and VRCZ was changed to 100 mg/day of itraconazole. Treatment was discontinued after three months, when infiltrates, as observed on chest X-rays, had

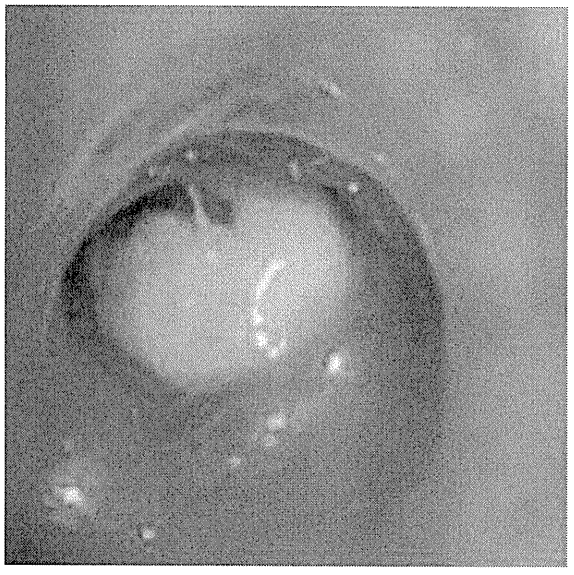

Fig. 2 Bronchoscopy findings. White plug-like polypoid region with hemorrhagic tendency is evident at the origin of the right bronchi B5.

completely disappeared (Fig. 1B). The titer of serum *Aspergillus* galactomannan antigen (ELISA) also decreased to 1.0 at the time of discharge. The patient has remained free of recurrent infection.

Mycological features of isolated Aspergillus *species*

Portions of colonies of the isolate grown on agar were examined by light microscopy and were morphologically characterized. Fig. 3 shows the appearance of a colony grown on modified Drigalski agar (Eiken Chemical Co., Ltd, Tokyo, Japan) for seven days at 37°C (Fig. 3A) and the light microscopy findings of lactophenol cotton-blue stained conidial head and conidia (Fig. 3B). The isolate was included in the *Aspergillus* section *Fumigati* based on macro- and micro-morphological characteristics, i.e., greenish-white colonies, uniseriate fruiting structures, phialides covering the upper half to two-thirds of the vesicle, and essentially smooth conidial surfaces.

Molecular techniques were employed for species identification. The genomic DNA of this isolate was prepared using Gentorukun® (Takara Bio Inc., Ltd, Otsu, Japan) and the β-tubulin gene was directly sequenced from PCR products using the primer pair Bt2a and Bt2b [6]. The PCR products were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3130ABI Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. We edited DNA sequences using ATGC Ver. 4 sequence assembly software (Genetyx Co., Tokyo, Japan). The GenBank database at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was then searched using the β-tubulin gene sequence and the BLAST algorithm. The results of the database search showed that the isolate had 100% similarity to sequences from *A. udagawae* or its teleomorph (accession numbers AB248294-248297 and DQ058392, respectively), with the number of hits ranging from 453/453 base pairs.

The morphological characteristics of the isolate and the results of the BLAST search of the strain were consistent

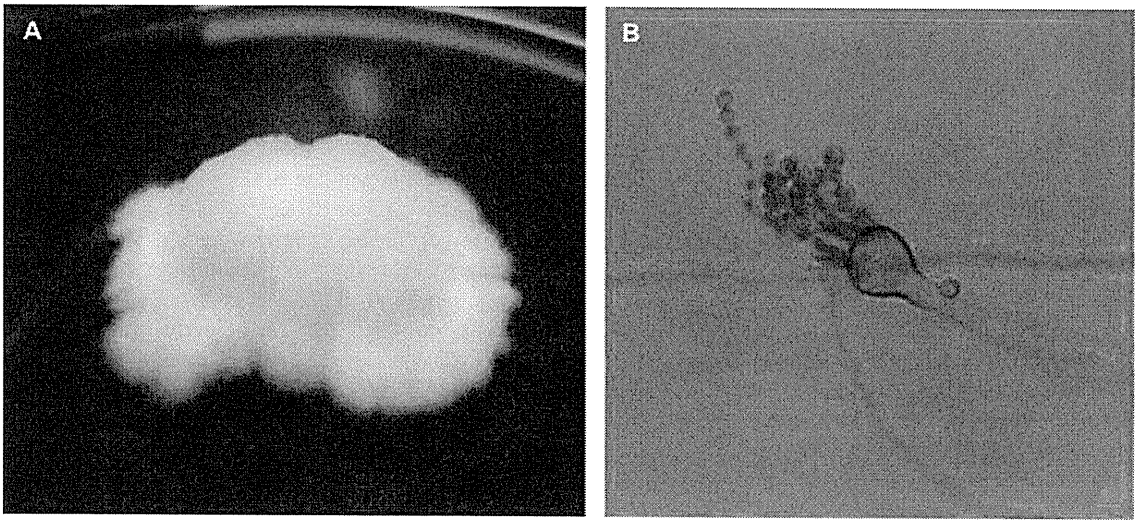

Fig. 3 Macroscopic and microscopic appearance of *Aspergillus udagawae* isolate in this case. Greenish white colonies were observed on modified Drigalski agar after 7 days at 37°C (A). Findings from light microscopy and lactophenol cotton-blue staining (B) magnification (× 400) indicate uniseriate fruiting structures, phialides covering the upper half to two-thirds of the vesicle, and essentially smooth conidia.

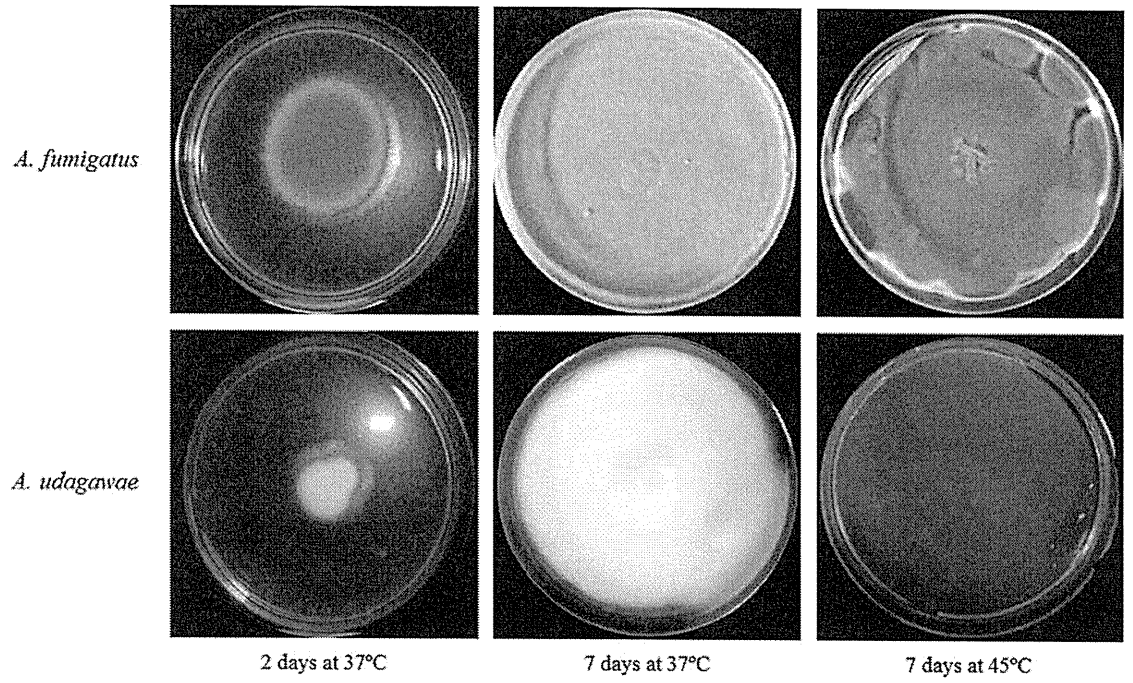

Fig. 4 Growth characteristics of *Aspergillus fumigatus* and *A. udagawae* on Czapek-Dox agar compared after 2 and 7 days at 37°C and after 7 days at 45°C. The macroscopic appearance of *A. udagawae* and *A. fumigatus* differs and *A. udagawae* could not grow at 45°C, unlike *A. fumigatus*.

with those of *A. udagawae*. Fig. 4 compares the appearance and colony features of *A. fumigatus* and *A. udagawae* grown on Czapek-Dox agar after 2 and 7 days at 37°C and after 7 days at 45°C. The appearance of *A. udagawae* and *A. fumigatus* differed, and the former was unable, unlike *A. fumigatus*, to grow at 45°C. The antifungal susceptibility of the isolate was also determined retrospectively using the Clinical and Laboratory Standards Institute M38-A2 broth microdilution method [7]. Minimum inhibitory concentrations (MICs) of itraconazole, VRCZ, and amphotericin B and minimum effective concentration (MEC) of micafungin were determined. The MICs of itraconazole, VRCZ and amphotericin B were 0.5, 0.5 and 2 μg/ml, respectively, and the MEC of micafungin was ≤ 0.015 μg/ml.

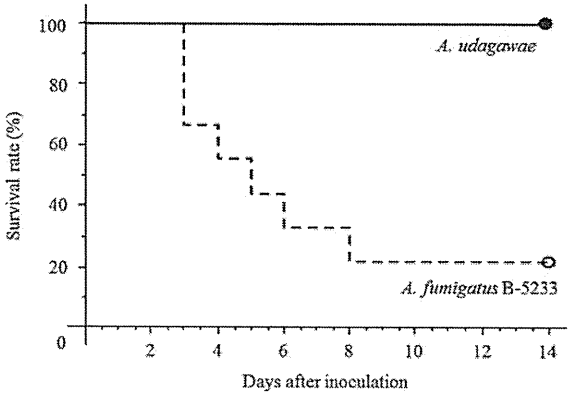

Fig. 5 Survival curves of immunosuppressed mice infected with *Aspergillus fumigatus* and *A. udagawae*. *A. udagawae* is statistically less virulent ($P \leq 0.01$) than *A. fumigatus* B-5233.

These data, compared with epidemiological cut-off values, indicated that the *A. udagawae* strain recovered from this patient is not susceptible to amphotericin B [8]. The pathogenesis of the isolate was evaluated using a mouse model and compared with that of *A. fumigatus* B-5233 strain (kindly provided by Dr K.J. Kwon-Chung, NIH, Bethesda, MD, USA). Eight-week-old female ICR mice (Charles River Breeding Laboratories, Shiga, Japan) were immunosuppressed by subcutaneous injections of 200 mg/kg of cortisone acetate (Sigma, Tokyo, Japan) on days −1, 0 and 1 and then intratracheally challenged on day 0 with 5×10^5 conidia of *A. fumigatus* B-5233 or *A. udagawae*. Survival was monitored as described with minor modification [9] for 14 days after the challenge and data are presented from one representative experiment using groups of nine mice each. All animal experiments were reviewed and approved by an ethical committee of Nagasaki University Animal Center. Survival curves were generated using the Kaplan-Meier method and statistical differences were evaluated by the log-rank test.

Fig. 5 shows that *A. udagawae* was statistically less virulent ($P \leq 0.01$) than *A. fumigatus* B-5233. We also

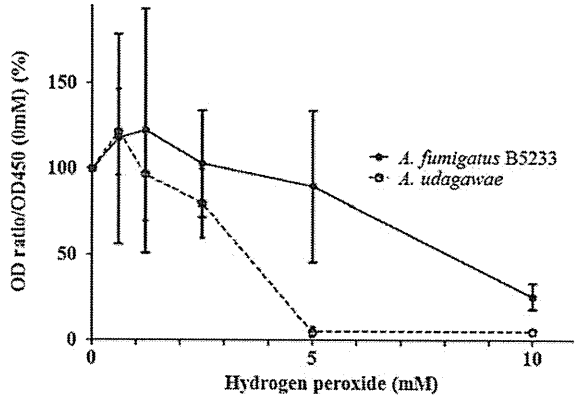

Fig. 6 Effect of hydrogen peroxide on *Aspergillus fumigatus* B-5233 and *A. udagawae* as evaluated by XTT assays. Metabolic activity of *A. udagawae* is significantly reduced compared with that of *A. fumigatus* B-5233.

examined and compared the sensitivity of the isolated *A. udagawae* strain and *A. fumigatus* B-5233 to hydrogen peroxide, a reactive oxygen species (ROS), by measuring the metabolic activity with the XTT assay as described with minor modification [5]. Briefly, the conidia of *A. fumigatus* B-5233 and *A. udagawae* were incubated with 0–20 mM hydrogen peroxide. The conidia were examined after 20 h and OD_{450} was measured. Data from two experiments that included triplicate measurements involving each concentration showed that the isolated *A. udagawae* strain was metabolically less active than *A. fumigatus* B-5233. This was statistically significant at the concentration of 5 mM ($P < 0.05$ by Student's *t*-test) (Fig. 6). These data indicate that the *A. udagawae* isolated in this case was more sensitive to ROS, which is produced by phagocytes as part of the primary defense system against pathogens.

Discussion

Advances in molecular techniques and tools have led to the understanding that *A. udagawae* and *A. fumigatus* are different species. The most important clinical feature of *A. udagawae* is that it is less sensitive to amphotericin B as compared to *A. fumigatus*. Since many laboratories do not characterize *Aspergillus* spp. beyond their morphological features, the potential prevalence of *A. udagawae* may be underestimated.

To our knowledge, this is the first report of bronchial aspergillosis caused by *A. udagawae*. The underlying diseases among patients infected with this fungus are chronic granulomatous disease and myelodysplastic syndrome [4]. These conditions usually worsen the immunocompromised status of patients, which in turn allows *A. udagawae* to cause invasive infections with poor prognoses [2–4]. The underlying disease of the patient in this case study was mild diabetes mellitus which may explain why her aspergillosis did not have a fatal outcome. One limitation of the diagnosis was that a biopsy sample could not be obtained from both the plugged and the peripheral-affected region (end side) due to a high hemorrhagic tendency. The confirmed diagnosis of invasive pulmonary aspergillosis could not be made due to lack of pathological evidence of *Aspergillus* hyphae in the tissue, including blood vessels. Biopsy of the bronchial tissue would have probably confirmed the possible invasion of the tissue by *A. udagawae*. However, the cytological examination and microbial culture proved that the bronchial plugged region was caused by *A. udagawae* and the diagnosis of bronchial aspergillosis was considered to be reasonable. Allergic bronchial pulmonary aspergillosis (ABPA) was another possibility. However, the patient did not have a history of bronchial asthma, eosinophilia, *Aspergillus* antibodies and elevated IgE, which ruled out this diagnosis as defined by Rosenberg [10].

Comparison of the mycological features of *A. udagawae* and *A. fumigatus* by Sugui *et al.* [5] showed that *A. udagawae* is less sensitive to amphotericin B, less virulent and more sensitive to hydrogen peroxide than *A. fumigates*. In addition, their colonies in culture are morphologically dissimilar; isolates of *A. udagawae* grow more slowly than *A. fumigatus*, and do not grow at 45°C. The characteristics of the *A. udagawae* strain isolated in this case were almost identical to these features. The next challenge will be to easily distinguish *A. udagawae* from *A. fumigatus* in the laboratory and clinical setting. We performed further molecular characterization of the isolate because the color of the colony was greenish white and the conidial surface appeared smooth. These findings differ from those of the typical *A. fumigatus*, which has blue green colonies and spinose conidia. However, some *A. fumigatus* isolates may display a greenish white appearance and it is actually unclear whether the appearance of conidia is smooth or spinose under a light microscope. Hence, molecular characterization was required for definite identification. The difference of maximum growth temperature is most significant and easily applied in the laboratory where molecular tools are unavailable. Furthermore, it is important to spread the awareness that bronchial aspergillosis can be caused by *A. udagawae* and that antifungal drugs other than amphotericin B are more appropriate.

In conclusion, this is the first report of a bronchial *A. udagawae* infection that was successfully treated by VRCZ. The characteristics of the *A. udagawae* strain isolated

from this patient were almost identical to those of the known *A. udagawae* strains [5].

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Medical Mycology August 2011, **49**, 581–587

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Original Articles

Diagnostic significance of *Aspergillus* species isolated from respiratory samples in an adult pneumology ward

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Although the diagnostic significance of isolating *Aspergillus* spp. from respiratory cultures has been studied in immunocompromised hosts with invasive pulmonary aspergillosis (IPA), little is known of such infections in immunocompetent patients with other forms of aspergillosis. In this study of adult pneumology ward patients, we examined the association between *Aspergillus* spp. and disease prevalence. Laboratory records from April 1998 to March 2009 were reviewed to identify patients with *Aspergillus* spp. in respiratory samples. Correlations between the isolated species and clinical characteristics of patients were evaluated. During the study period, 165 *Aspergillus* spp. isolates were detected in the respiratory cultures of 139 patients. Of these patients, 62 (45%) were colonized with *Aspergillus* spp. and displayed no clinical symptoms of aspergillosis, while 77 (55%) had a form of pulmonary aspergillosis, characterized as either chronic necrotizing pulmonary aspergillosis (CNPA) (48%), aspergilloma (29%), IPA (13%), or allergic bronchopulmonary aspergillosis (ABPA) (10%). The dominant species were *Aspergillus fumigatus* (41%), *A. niger* (32%), and *A. versicolor* (12%). *A. fumigatus* was most commonly isolated in patients with IPA, aspergilloma, and CNPA, whereas *A. niger* was the dominant species in colonized patients and those with ABPA. Isolation of an *Aspergillus* spp. from respiratory samples does not confirm it as the etiologic pathogen because airway colonization by *Aspergillus* spp. is a common feature in several chronic lung diseases. Repeated isolation of the identical *Aspergillus* species and detection of anti-*Aspergillus* antibodies and/or *Aspergillus* antigens in sera are needed to determine the isolate represents the etiologic agent of disease.

Keywords CNPA, CCPA, Aspergilloma, IPA, colonization

Introduction

Members of the genus *Aspergillus* are ubiquitous saprophytic fungi, and due to their widespread presence in the environment, the average person may inhale hundreds of *Aspergillus* conidia per day [1]. Conidia are typically removed from the respiratory tract by mucociliary clearance and phagocytosis by alveolar macrophages, while germinating spores and hyphae are attacked by polymorphonuclear neutrocytes through degranulation and the release of oxidants [2]. Despite these effective clearance mechanisms for the elimination of inhaled conidia from the respiratory tracts of healthy individuals, *Aspergillus* conidia are capable of colonizing injured lung tissue and epithelia. Although such colonization often has no clinical consequences, *Aspergillus* conidia can cause a variety of

Received 21 September 2010; Received in final revised form 3 December 2010; Accepted 12 December 2010

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DOI: 10.3109/13693786.2010.548084

clinical manifestations depending on the immune status of the host [3,4]. For example, allergic bronchopulmonary aspergillosis (ABPA), which encompasses hypersensitivity reactions to *Aspergillus* antigens, can develop in atopic patients [5,6], while aspergilloma is mainly observed in patients with chronic cavitary lung disease. Other forms of aspergillosis include chronic necrotizing pulmonary aspergillosis (CNPA), which is seen in mildly immunocompromised patients and those individuals with chronic lung diseases [7], and invasive pulmonary aspergillosis (IPA), which is the most serious lung infection due to *Aspergillus* spp. and typically occurs only in severely immunocompromised patients [8].

Isolation of *Aspergillus* spp. from respiratory samples of a patient with suspected aspergillosis does not necessarily indicate that the identified species is the etiologic agents of disease. Although the diagnostic significance of the isolation of *Aspergillus* spp. from respiratory cultures has been extensively examined in immunocompromised hosts who develop IPA [9–13], little is known in respect to the presence of *Aspergillus* spp. in the respiratory tracts of immunocompetent or mildly immunocompromised patients with other forms of aspergillosis [14–16]. Here, we conducted a retrospective study to evaluate the clinical significance of *Aspergillus*-positive culture results of respiratory samples from adult patients in a pneumology ward.

Materials and methods

The microbiology laboratory records of the Pneumology Department of Nagasaki University Hospital, Japan, from April 1998 to March 2009 were reviewed to identify all patients who had *Aspergillus* spp. isolated in culture from respiratory samples. A total of 350–500 patients including 150–200 new patients per year are admitted to this department, with lung cancer being the most common pulmonary disease. The respiratory samples, which included sputum, endotracheal aspirates, and bronchoalveolar lavage fluid, were obtained as a part of a diagnostic work-up for respiratory disease. Samples were immediately inoculated onto Sabouraud dextrose agar and incubated for 10 days at 30°C. Filamentous fungi recovered in culture were transferred to potato dextrose agar and incubated for 2–3 days at 30°C. The isolates were identified based upon macroscopic and microscopic morphological characteristics following standard mycological procedures. For each patient with a positive sample for *Aspergillus*, the species and clinical characteristics of the patient were recorded. When the same *Aspergillus* species was isolated on more than one occasion from the same patient, it was counted only once.

Aspergilloma was diagnosed when a characteristic fungus ball was detected within a lung cavity by chest radiograph or computed tomography and a positive serological test (antibody detected) was obtained or when an *Aspergillus* species was isolated from respiratory samples. CNPA was diagnosed when chest radiographs showed a cavitary pulmonary lesion with evidence of paracavity infiltrates or cavitary change developing over a time-frame of several weeks to several months in patients with chronic pulmonary symptoms, elevated inflammatory markers, and positive serological tests (antigen/antibody detected) or isolation of *Aspergillus* spp. from respiratory samples [17]. IPA was recorded when chest radiographs showed acute and extensive pneumonia or nodular pneumonia resistant to broad-spectrum antibiotics in immunocompromised patients, and respiratory samples yielded *Aspergillus* spp. without pathogenic bacteria. ABPA was diagnosed according to the criteria of Rosenberg [5]. Serological studies including *Aspergillus* antibody and antigen tests were performed when the patients were suspected of having pulmonary aspergillosis. Patients were diagnosed as having airway colonization when *Aspergillus* spp., were isolated from respiratory samples without clinical or histological evidence of aspergillosis. All of the charts were reviewed by TT, KI, MT, TT, TM, and HK as review committee. All members of the chart review committee discussed each case if there was an incorrect or unclear diagnosis of aspergillosis. All data was reviewed by all authors.

Results

During the 11-year study period, 165 isolates of *Aspergillus* spp. were detected in cultures inoculated with the respiratory samples of 139 patients. Of these patients, 62 (45%) were colonized with *Aspergillus* spp. but displayed no clinical symptoms of aspergillosis, while the remaining 77 (55%) had a form of pulmonary aspergillosis, which could be classified as CNPA (48%), aspergilloma (29%), IPA (13%), or ABPA (10%). The clinical characteristics of the patients are summarized in Table 1.

The patients with CNPA or aspergilloma displayed a high occurrence of associated chronic lung disease, including chronic obstructive pulmonary disease (COPD), nontuberculous mycobacteriosis (NTM), pulmonary tuberculosis, lung cancer, and bullae or a history of pneumothorax, interstitial pneumonia, and pneumoconiosis. In addition, the majority of patients with CNPA suffered from a mild systemic immunosuppressive condition, such as diabetes mellitus, solid-organ cancer, chronic liver disease, and corticosteroid or cytotoxic drug use. In patients with IPA, systemic immunosuppression due to hematologic malignancy, corticosteroid therapy, or chemotherapy was the major predisposing factor, while patients with ABPA often displayed bronchial asthma (88%) and/or other atopic diseases (63%). Of the 62 patients with *Aspergillus* colonization, nearly all had an associated chronic lung disease, such as tuberculosis, COPD, lung cancer, NTM, interstitial

Table 1 Clinical characteristics of patients with *Aspergillus*-positive cultures from respiratory samples.

Characteristic[a]	No. (%) of patients, according to clinical condition				
	CNPA (n = 37)	Aspergilloma (n = 22)	IPA (n = 10)	ABPA (n = 8)	Colonization (n = 62)
Age in years, range (mean)	19–83 (64.7)	46–84 (68.7)	18–83 (58.8)	27–80 (51.6)	20–85 (61.8)
Sex (male/female)	29/8	15/7	7/3	4/4	26/36
Underlying lung disease					
COPD	14 (38)	9 (41)	1 (10)		11 (18)
Non-tuberculous mycobacteriosis	13 (35)	1 (5)		1 (13)	10 (16)
Tuberculosis	9 (24)	9 (41)		1 (13)	14 (23)
Lung cancer survivor	5 (14)	2 (9)		1 (13)	11 (18)
Bullae or pneumothorax	4 (11)	3 (14)			4 (6)
Interstitial pneumonia	2 (5)	1 (5)	1 (10)		10 (16)
Pneumoconiosis	2 (5)	1 (5)			2 (3)
Bronchial asthma	1 (3)			7 (88)	1 (2)
Other lung diseases		2 (9)	2 (20)		8 (13)
Immunosuppressive condition					
Solid organ cancer	6 (16)		1 (10)		4 (6)
Hematologic malignancy	1 (3)		5 (50)		4 (6)
Diabetes mellitus	11 (30)		2 (20)		2 (3)
Chronic liver disease	3 (8)		2 (20)		2 (3)
Chronic kidney disease	1 (3)				3 (5)
Collagen vascular disease					10 (16)
Atopic disease				5 (63)	
Other diseases	2 (5)				
Corticosteroid use	3 (8)		5 (50)		12 (19)
Cytotoxic drug use			5 (50)		2 (3)
Bone marrow transplant			1 (10)		
In-hospital mortality	6 (16)	1 (5)	8 (80)	0 (0)	1 (2)

CNPA, chronic necrotizing pulmonary aspergillosis; IPA, invasive pulmonary aspergillosis; ABPA, allergic bronchopulmonary aspergillosis; COPD, chronic obstructive pulmonary disease.
[a]A patient may have more than one characteristic.

pneumonia, and/or an immunosuppressive condition induced by corticosteroid use, collagen vascular disease, solid-organ cancer other than lung, hematologic malignancy, or diabetes mellitus. In-hospital mortality was highest in patients with IPA (80%), whereas it was relatively low in patients with CNPA (16%), aspergilloma (5%), and *Aspergillus* colonization (2%). None of the patients with ABPA died during their hospitalization period.

We determined the distribution of *Aspergillus* spp. among the 165 isolates from the respiratory cultures of 139 patients (Table 2). The two dominant species of *Aspergillus* were *A. fumigatus* (41%) and *A. niger* (32%), with *A. versicolor* (12%), *A. terreus* (6%), *A. flavus* (5%), *A. nidulans* (2%), *A. sydowii* (1%), and unidentifiable *Aspergillus* spp. (0.6%) accounting for the remaining isolates. When the patient data between 1998 and 2004 were compiled, *A. fumigatus* was the most commonly isolated species (58.2%), followed by *A. niger* (20.9%), *A. flavus* (10.4%), *A. versicolor* (6.0%), and *A. terreus* (4.5%). However, between 2005 and 2009, the incidence

Table 2 *Aspergillus* species associated with disease based on positive culture results.

Aspergillus species	No. (%) of positive culture results, according to clinical condition					
	CNPA (n = 50)	Aspergilloma (n = 25)	IPA (n = 11)	ABPA (n = 10)	Colonization (n = 69)	Total (n = 165)
A. fumigatus	27 (54)	17 (68)	9 (82)	3 (30)	11 (16)	67 (41)
A. niger	12 (24)	3 (12)	1 (9)	4 (40)	33 (48)	53 (32)
A. versicolor	3 (6)	1 (4)		2 (20)	14 (20)	20 (12)
A. terreus	5 (10)			1 (10)	4 (6)	10 (6)
A. flavus	2 (4)	3 (12)	1 (9)		3 (4)	9 (5)
A. nidulans	1 (2)	1 (4)			1 (1)	3 (2)
A. sydowii					2 (3)	2 (1)
Not identified					1 (1)	1 (0.6)

CNPA, chronic necrotizing pulmonary aspergillosis; IPA, invasive pulmonary aspergillosis; ABPA, allergic bronchopulmonary aspergillosis.

of *A. fumigatus* isolation decreased by approximately half (28.6%), whereas that of *A. niger* isolation increased approximately two-fold (39.8%), with *A. versicolor* (16.3%) and *A. terreus* (7.1%) again being the next most common isolates (Fig. 1).

To gain a better understanding of the diagnostic significance of the presence of *Aspergillus* spp. in respiratory samples, we examined the correlation between the identified species and the clinical characteristics of patients with regard to disease. It was revealed that *A. fumigatus* was the most commonly isolated species in patients with IPA (82%), aspergilloma (68%), and CNPA (54%), while *A. niger* was the second most prevalent isolate in patients with these diseases. In patients with ABPA, *A. niger* was the most common isolate (40%), followed by *A. fumigatus* (30%). *A. niger* was also the most common isolate (48%) in patients who were asymptomatic for aspergillosis, followed by *A. versicolor* (20%) and *A. fumigatus* (16%). Interestingly, with regard to colonization by *Aspergillus* species, 70% (14/20) and 62% (33/53) of cases involving isolation of *A. versicolor* and *A. niger* isolation, respectively, were considered as asymptomatic, whereas only 16% (11/67) of cases of *A. fumigatus* isolation were not associated with disease.

Finally, we examined the respiratory tract colonization profiles and patterns among the 139 patients positive for *Aspergillus* spp. In most patients (83%), only a single *Aspergillus* spp. was isolated from respiratory tract samples, whereas two or more species were isolated in the remaining patients (17%). In 17 of these 23 patients, a synchronous isolation pattern was observed, which was characterized by isolation of two or more species in the same sample or different samples obtained within a one-month period (Table 3). The remaining six patients displayed a metachronous isolation pattern, in which different samples obtained with an interval of over one month was revealed (Table 4). The combination of *A. fumigatus* and *A. niger* was the most common profile for both the synchronous and metachronous isolation patterns of the observed species combinations.

Discussion

Although the genus *Aspergillus* consists of approximately 200 species [2,18], only a few are considered human pathogens and generally require immunocompromised hosts to cause disease. Among *Aspergillus* spp., *A. fumigatus* is the primary causative agent of human infections, followed by *A. flavus*, *A. terreus*, *A. niger*, and *A. nidulans* [8,19–26]. In the present retrospective study, *A. fumigatus* was the most commonly isolated species from respiratory samples, followed by *A. niger*, *A. versicolor*, *A. terreus*, *A. flavus*, *A. nidulans*, and *A. sydowii*. However, our analysis of laboratory records and clinical disease revealed that the isolation of a particular *Aspergillus* spp. does not confirm it is the etiologic pathogen of the patient. The diagnostic value of *Aspergillus* spp. in respiratory samples is not straightforward, principally because of difficulties distinguishing colonization from disease. In our series, 42% (69 of 165) of the isolated *Aspergillus* spp. were found in cases representing colonization, and the most common colonizing species was *A. niger*, followed by *A. versicolor*, *A. fumigatus*, *A. terreus*, *A. flavus*, *A. sydowii*, and *A. nidulans*. Although *A. niger* is less virulent than *A. fumigatus* and *A. flavus*, it can occasionally cause IPA, CNPA, or ABPA, and may colonize respiratory tracts of patients with chronic lung diseases [8,23,24]. From our analyses, *A. niger* was most frequently associated with patients diagnosed with ABPA (40%), but for all other forms of pulmonary aspergillosis, *A. fumigatus* was the dominant isolate. Although *A. fumigatus* is the most pathogenic *Aspergillus* spp., it may also colonize respiratory tracts without leading

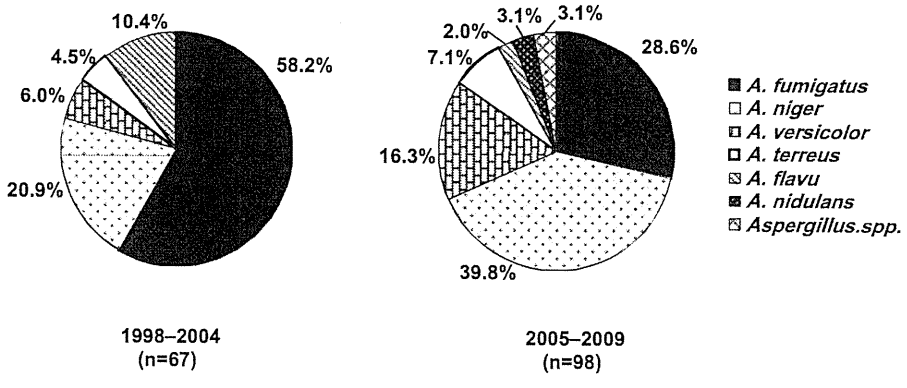

Fig. 1 Comparison of *Aspergillus* spp. isolated during 1998–2004 and 2005–2009 from adults in a pneumology ward. *A. fumigatus* was the most commonly isolated species between 1998 and 2004; however, between 2005 and 2009 its frequency of isolation had decreased by approximately half, while *A. niger* increased approximately two-fold to become the most commonly isolated species.

Table 3 Synchronously isolated *Aspergillus* species according to clinical condition.

Aspergillus species	No. of patients, according to clinical condition					
	CNPA (n = 7)	Aspergilloma (n = 1)	IPA (n = 1)	ABPA (n = 2)	Colonization (n = 6)	Total (n = 17)
A. fumigatus + *A. niger*	3	1		1	2	7
A. niger + *A. versicolor*	1				2	3
A. fumigatus + *A. terreus*	1					1
A. fumigatus + *A. flavus*			1			1
A. niger + *A. flavus*					1	1
A. niger + *A. terreus*				1		1
A. niger + *A. sydowii*					1	1
A. terreus + *A. nidulans*	1					1
A. fumigatus + *A. niger* + *A. versicolor*	1					1

CNPA, chronic necrotizing pulmonary aspergillosis; IPA, invasive pulmonary aspergillosis; ABPA, allergic bronchopulmonary aspergillosis.

to clinical symptoms of aspergillosis, as was observed in 16% of cases.

The reported frequency of *Aspergillus* spp. colonization ranges from 36–91% of clinical cases, depending on the patient population studied [9–16]. Perfect *et al.* [11] reported that approximately 50% of *Aspergillus*-positive cultures represented colonization, with *A. fumigatus* (63%) the dominant colonizing species, followed by *A. niger* (14%), *A. flavus* (9%), *A. nidulans* (1%), *A. terreus* (1%), and other species (4%). Although the general trend for the colonizing species in our data was similar, the prevalence of *A. fumigatus* (41%) and *A. niger* (32%) was approximately equal. The difference in the incidence of colonizing species between the two studies is considered to be mainly a result of the study periods and patients. Our data were obtained between 1998 and 2009, whereas Perfect *et al.* [11] conducted their surveillance in 1995. As our data for 1998 and 2004 more closely resembled that of Perfect *et al.* [11] with regard to the distribution of *A. fumigatus* (58.2%) and *A. niger* (20.9%). It appears that *A. niger* is becoming more prevalent in respiratory tract samples, a speculation that is consistent with a reported increase in the frequency of *A. niger* isolation in recent years [27–29]. Colonization may represent transient passage in the airway, as long-term benign carriage is typically observed in patients with localized structural or functional pulmonary deficits. However, it may be a warning sign preceding overt *Aspergillus* infection [14,15].

Table 4 Metachronously isolated *Aspergillus* species according to clinical condition.

Aspergillus species	Clinical condition (No. of patients)
A. fumigatus → *A. niger*	CNPA (n = 2)
A. niger → *A. fumigatus*	CNPA (n = 1)
A. niger → *A. fumigatus* → *A. terreus* → *A. versicolor*	CNPA (n = 1)
A. niger → *A. fumigatus* → *A. nidulans*	Aspergilloma (n = 1)
A. terreus → *A. fumigatus*	Colonization (n = 1)

CNPA, chronic necrotizing pulmonary aspergillosis.

CNPA, which was originally reported by Binder *et al.* [7], has been widely used to characterize a syndrome complex consisting of slowly progressive cavitary lung disease, chronic respiratory symptoms, and the presence of precipitating antibodies to *Aspergillus* spp. A few reports related to CNPA have described direct invasion of pulmonary parenchyma by hyphal elements [7,23,30]. However, the majority of reports have not found clear evidence of parenchymal invasion despite progressive tissue damage. Denning *et al.* [31] proposed that chronic cavitary pulmonary aspergillosis (CCPA) accounts for cases in which there is formation and expansion of multiple cavities over time. Furthermore, they proposed that the term CNPA be reserved for cases in which hyphal invasion of tissue is demonstrated, such as the subacute form of IPA. The majority of our patients with CNPA may have been diagnosed with CCPA, because the pathological appearance of hyphal invasion had not been examined. In our patients with CNPA, *A. fumigatus* was the most commonly isolated species (54%), followed by *A. niger* (24%), *A. terreus* (10%), *A. versicolor* (6%), *A. flavus* (4%), and *A. nidulans* (2%). This result is consistent with that of Perfect *et al.* [11] who reported that the most common *Aspergillus* species identified in patients with CNPA was *A. fumigatus* (80%), followed by *A. niger* (10%), *A. flavus* (2%), and other species (8%).

The profile of *Aspergillus* spp. isolated from patients with aspergilloma was similar to that from patients with CNPA, and consisted of *A. fumigatus* (68%), *A. niger* (12%), *A. terreus* (12%), *A. versicolor* (4%), and *A. nidulans* (4%). Perfect *et al.* [11] reported that the most common *Aspergillus* species identified in patients with aspergilloma was *A. fumigatus* (69%), followed by *A. niger* (13%), *A. flavus* (2%), and other species (5%).

The majority of IPA is caused by *A. fumigatus*, with the second most frequent pathogenic species being *A. flavus* and, to a lesser extent, *A. niger* and *A. terreus* [8,32]. In our retrospective study, the most common *Aspergillus* species identified in patients with IPA was *A. fumigatus* (80%), followed by *A. niger* (9%) and *A. flavus* (9%). Perfect *et al.* [11] reported that *A. fumigatus* (67%) was most commonly isolated, followed by *A. flavus* (16%), *A. niger* (5%), *A. terreus* (3%), and *A. nidulans* (1%).

ABPA is an allergic pulmonary disorder caused by hypersensitivity to *Aspergillus* spp., and *A. fumigatus* is the primary causal organism. In our series, the isolated *Aspergillus* spp. from patients with ABPA were *A. niger* (40%), *A. fumigatus* (30%), *A. versicolor* (20%), and *A. terreus* (10%). Since we did not test allergic reactions to non-*A. fumigatus Aspergillus* spp., isolates other than *A. fumigatus* could not be confirmed as the etiologic antigen to ABPA. However, ABPA due to *A. flavus*, *A. nidulans*, *A. terreus* or *A. niger* has been reported [33–35]. Recently, Benndorf *et al.* [36] identified IgE antibodies that exclusively recognize spore extracts from *A. versicolor* in sera from patients, and also found a relationship between increased spore concentration in indoor air or visible mould affection and a positive reaction of sera to *A. versicolor*. As the isolation of *A. versicolor* in respiratory samples is generally considered as colonization [37–40], the possibility that it is an etiologic agent for aspergillosis, including ABPA, cannot be denied.

Two limitations of this study warrant mentioning. First, as histopathological observations were not available for most patients, we could not differentiate between CNPA and other forms of chronic pulmonary aspergillosis, including CCPA. Second, since immunological tests, such as IgG or IgE antibody titer and antigen tests for non- *fumigatus Aspergillus* spp. were not available, isolates other than *A. fumigatus* could not be confirmed as the etiologic agent of disease in chronic pulmonary aspergillosis including ABPA.

In conclusion, the results of our study show that isolation of *Aspergillus* spp. from respiratory samples does not confirm that they represent the etiologic pathogen, because airway colonization by *Aspergillus* spp. is a common feature in patients with chronic lung disease. Even if an *Aspergillus* is isolated in a patient with clinically diagnosed aspergillosis, the colonizing species may not be associated with mycotic disease. The examined clinical, radiological, and microbiological data from 139 patients positive for *Aspergillus* spp. isolates did not allow differentiation between infection and colonization in several cases. It is therefore considered that repeated isolation of the identical *Aspergillus* species and detection of anti-*Aspergillus* antibodies and/or *Aspergillus* circulating antigens in sera are needed to conclude that an isolated species represents the etiological organism in immunocompetent or mildly immunocompromised individuals.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 11 January 2011.

Experimental and Molecular Pathology 91 (2011) 614–621

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Imaging mass spectrometry analysis reveals an altered lipid distribution pattern in the tubular areas of hyper-IgA murine kidneys

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ARTICLE INFO

Article history:
Received 7 July 2011
Available online xxxx

Keywords:
Imaging mass spectrometry
Phosphatidylcholine
Urine
Lipid
IgA
Nephropathy
Urinary stagnation
Molecular distribution
Molecular imaging
Deposition

ABSTRACT

Immunoglobulin A (IgA) nephropathy is the most common glomerular disease worldwide. To investigate the pathogenesis of this renal disease, we used animal models that spontaneously develop mesangioproliferative lesions with IgA deposition, which closely resemble the disease in humans. We analyzed the molecular distribution of lipids in hyper-IgA (HIGA) murine kidneys using matrix-assisted laser desorption/ionization-quadrupole ion trap-time of flight (MALDI-QIT-TOF)-based imaging mass spectrometry (IMS), which supplies both spatial distribution of the detected molecules and allows identification of their structures by their molecular mass signature. For both HIGA and control (Balb/c) mice, we found two phosphatidylcholines, PC(16:0/22:6) and PC(18:2/22:6), primarily located in the cortex area and two triacylglycerols, TAG(16:0/18:2/18:1) and TAG(18:1/18:2/18:1), primarily located in the hilum area. However, several other molecules were specifically seen in the HIGA kidneys, particularly in the tubular areas. Two HIGA-specific molecules were O-phosphatidylcholines, PC(O-16:0/22:6) and PC(O-18:1/22:6). Interestingly, common phosphatidylcholines and these HIGA-specific ones possess 22:6 lipid side chains, suggesting that these molecules have a novel, unidentified renal function. Although the primary structure of the HIGA-specific molecules corresponding to *m/z* 854.6, 856.6, 880.6, and 882.6 remained undetermined, they shared similar fragmentation patterns, indicating their relatedness. We also showed that all the HIGA-specific molecules were derived from urine, and that artificial urinary stagnation—due to unilateral urethral obstruction—caused HIGA-specific distribution of lipids in the tubular area.

Introduction

Histopathological findings have provided a significant amount of information on nephropathies and have been used to solve their underlying mechanisms, such as IgA deposition in IgA nephropathy (Muda et al., 1995). In recent years, the emergence of molecular imaging techniques such as green fluorescent protein labeling and immunohistochemistry have expanded the practical applications available to researchers (Drummond and Allen, 2008; Grunkin et al., 2011). Although the techniques for histopathology have been useful in investigating the morphology and distribution of various defects in tissues, conventional techniques such as electron microscopy have failed to identify low-molecular-weight compounds. Moreover, while new systematic approaches (e.g., proteomics and metabolomics using mass spectrometry (MS)) have enabled the identification of various kinds of molecular species and have contributed to a more detailed understanding of the etiology of the disease, as well the discovery of new biomarkers (Baronas et al., 2007; Mimura et al., 1996; Yasuda et al., 2006; Yoshioka et al., 2009; Zhang et al., 2008), these approaches lose the distributional information.

Matrix-assisted laser desorption/ionization-quadrupole ion trap-time of flight (MALDI-QIT-TOF)-based imaging mass spectrometry (IMS) is a technique that supplies both the spatial distribution of the detected molecules and allows the identification of their structures by their molecular mass signature. More recently, the resolution of MALDI-QIT-TOF-IMS has been refined to microscopic level, thereby enabling an analysis of microscopic lesions that conventional approaches have not been able to easily examine (Setou and Kurabe, 2011). Willems et al. recently reported the usefulness of the IMS technology for grading myxoid sarcoma by clustering of the biomolecular signatures in particular lipid compositions (Willems et al., 2010).

The relationship between lipid composition and kidney diseases is not well understood. Hence, we investigated the distribution of lipid compositions in the kidneys of HIGA mice—well-recognized murine

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doi:10.1016/j.yexmp.2011.07.002

model for IgA nephropathy (Muso et al., 1996)—using MALDI-QIT-TOF-IMS and analyzed the physiological significance of the molecules detected by it.

Materials and methods

Chemicals

All general chemicals used in this study were purchased from Wako Chemicals (Tokyo, Japan), unless otherwise indicated, and were of the highest purity available. Ultra pure water dispensed by a Milli-Q water system (Millipore, Bedford, MA, USA) was used for the preparation of buffers and solvents.

Animals and sample preparation

All the experiments on the mice were conducted according to the protocols approved by the Animal Care and Use Committee, Nagasaki University, School of Medicine. Kidneys were obtained from 28-week-old Balb/c mice and HIGA mice (Charles River Japan, Kanagawa, Japan), and urine was collected in a cage designed to prevent feces–urine contact (Nalge Nunc International, Tokyo, Japan), as previously described (Kurashige et al., 2008). The tissue samples and urine were immediately frozen and stored at −80 °C until use.

Tissue slice preparation

Tissue slice preparation for imaging mass spectrometry was performed as previously described (Hayasaka et al., 2008; Sugiura and Setou, 2009). Briefly, the frozen intact tissues were sectioned at −20 °C in a cryomicrotome (CM 3050; Leica Microsystems, Wetzlar, Germany) to obtain 5-μm-thick sections, and the frozen slices were then thaw-mounted on indium tin oxide (ITO)-coated glass slides (Bruker Daltonics, Leipzig, Germany). Matrix was coated on the slices by spraying them with 100 μl of 2,5-dihydroxybenzoic acid (Bruker Daltonics) solution (50 mg/ml in 70% methanol/0.1% trifluoroacetic acid) using a 0.2-mm nozzle caliber airbrush (Procon Boy FWA Platinum; Mr. Hobby, Tokyo, Japan). After drying, the ITO slide was adhered to a mass spectrometer target plate with double-sided conductive adhesive tape to facilitate electrical conduction. Positional information for each section was obtained by scanning the section with a chemical inkjet printer CHIP-1000 (Shimadzu Corporation, Kyoto, Japan) prior to MALDI-QIT-TOF-MS analysis.

Extraction of lipids from tissue and urine

Tissues were crudely ground using clean spatulas and further shredded using a sonicator in an approximately 20-fold volume of chloroform-methanol (2:1) in glass tubes on ice. The mixture was

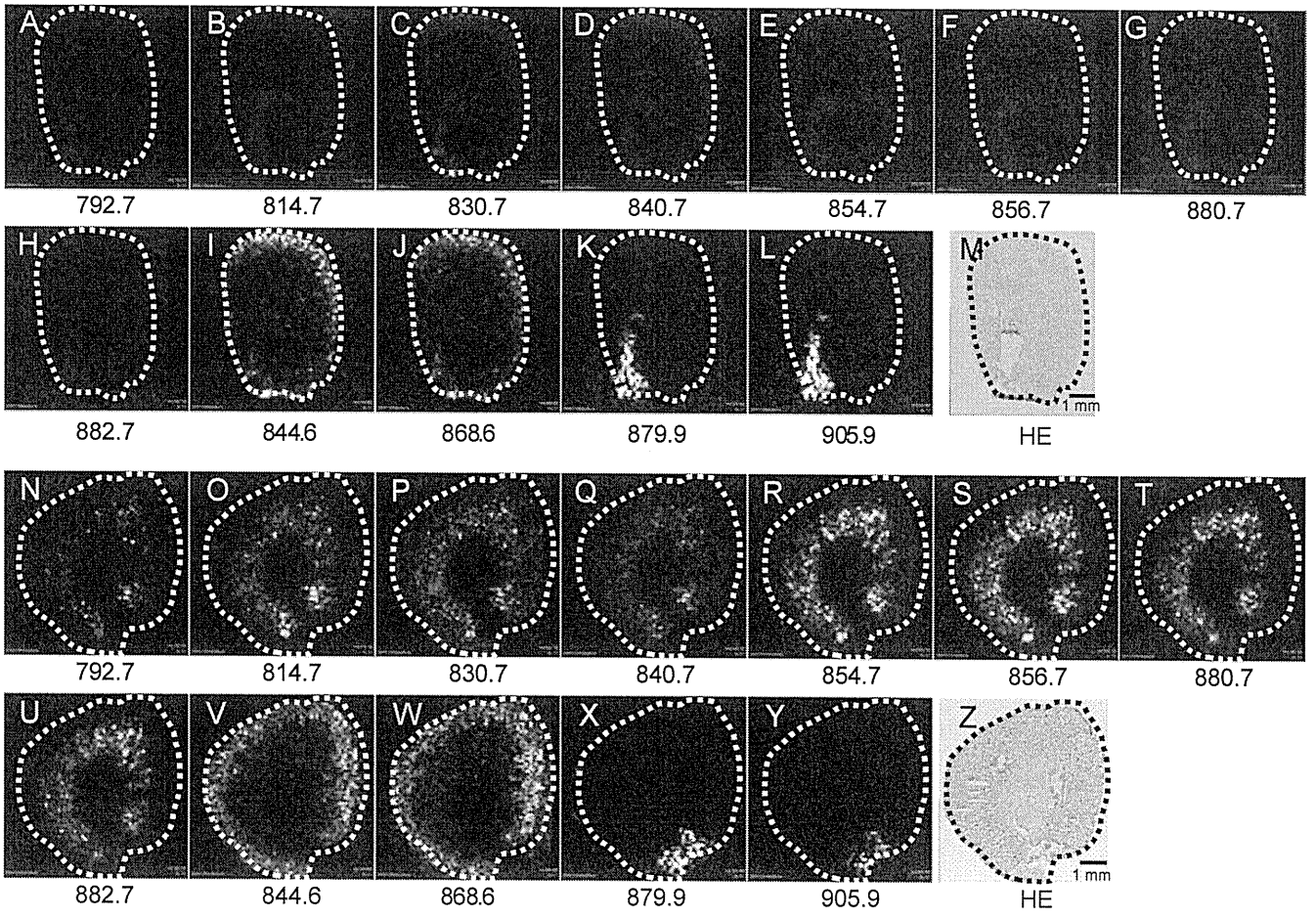

Fig. 1. Eight mass peaks are dominant in the kidneys of HIGA mouse in mass images of kidneys. Significant peaks, *m/z* 792.6, *m/z* 814.6, *m/z* 830.6, *m/z* 840.6, *m/z* 854.6, *m/z* 856.6, *m/z* 880.6, and *m/z* 882.6, are detected in HIGA kidneys (N–U), but absent in the control (A–H). However, 2 significant mass peaks are seen in the peripheral (cortex) area (I,J,V,W), and 2 other significant mass peaks are seen in the hilum area (K,L,W,Y) in both HIGA and control kidneys. The corresponding HE images of the control (M) and HIGA (Z) are also shown. Scale bars indicate a length of 1 mm.