Research Article

Candida albicans and C. tropicalis Isolates from the Expired Breathes of Captive Dolphins and Their Environments in an Aquarium

Hideo Takahashi, Keiichi Ueda, Eiko Nakagawa Itano, Makio Yanagisawa, Yoshiteru Murata, Michiko Murata, Takashi Yaguchi, Masaru Murakami, Katsuhiko Kamei, Tomo Inomata, Hirokazu Miyahara, Ayako Sano, and Senzo Uchida

1 Medical Mycology Research Center, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba, 260-8673, Japan
2 Aquatic Mammal Section, Okinawa Churaumi Aquarium, 424 Ishikawa, Motobu-cho, Kunigami-gun, Okinawa, 905-0206, Japan
3 Laboratory of Applied Immunology, Department of Pathological Sciences, Center of Biological Sciences, State University of Londrina, 86051-970 Londrina, PR, Brazil
4 Laboratory of Molecular Biology, Department of Veterinary Medicine, Azabu University School of Veterinary Medicine, 1-17-71, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa, 252-5201, Japan
5 Laboratory of Experimental Animals, Department of Veterinary Medicine, Azabu University School of Veterinary Medicine, 1-17-71, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa, 252-5201, Japan

 Correspondence should be addressed to Ayako Sano, aya1@faculty.chiba-u.jp

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Genotypes of Candida spp. isolated from exhalation of 20 dolphins, 11 water samples from captive pools, and 24 oral cavities of staff members in an aquarium using a combination of multiple drug resistance 1 gene (MDR1) and the internal transcribed spacer (ITS) 1 5.8s-ITS 2 regions of ribosomal RNA gene (ITS rDNA) sequences were studied. The holding ratios of the dolphins, captive pools, and staff members were 70, 90, and 29%, respectively. Isolated pathogenic yeast species common to the dolphins and environments were Candida albicans and C. tropicalis. Identical genotypes in both Candida spp. based on the combination of MDR1 and ITS rDNA were found in some dolphins, between a dolphin and a staff, among dolphins and environments, and among environments. The results indicated the diffusion and exchange of pathogenic yeasts at the aquarium among dolphins and environments. The isolates at the aquarium showed higher rates of resistance to azole antifungals compared to reference isolates.

1. Introduction

Mycotic diseases in delphinoids sometimes cause fatal outcomes or difficulties for the cares of animals [1–3]. Lobomycosis caused by Lacazia lobo (formerly Loboa lobo) is listed as the most famous mycosis in dolphins and zoonotic mycosis [1–12]. Apart from lobomycosis, Aspergillus spp. [1–3, 12–15], Candida albicans and other Candida spp. [16–20], Chladosporium sp. [21] and Chaladophilophora bantiana [3], Cryptococcus neoformans [18–20, 22–24], Fusarium spp. [25], Sporothrix schenckii [26], Trichophyton sp. [27], Trichosporon sp. [1–3], and zygomycetes, [28–30] which are common to human fungal infections, have also been documented as causative agents for pulmonary, disseminated and cutaneous fungal infections in the animals [1–3]. Highly pathogenic mycoses caused by Coccidioides immitis [31], Histoplasma capsulatum [32], and Blastomyces dermatitidis [33] have also been reported.

Besides being highly pathogenic, the above fungal species were isolated from exhalation, although the findings do not support correlations between mycoses—fungal pneumonia and/or disseminations and these organisms [3, 34]. In fact, even in healthy dolphins, pathogenic fungal species were isolated from exhalation [35]. Most species of pathogenic fungi
isolated from exhalation were environmental contaminants while *C. albicans* and other *Candida* spp. existed as normal fungal residents of mucous membranes [3, 35]. Furthermore, these human pathogenic yeast species were isolated from more than 70% of captive dolphins and environmental water samples [19].

According to Buck [19] and Dunn et al. [17], there is no transmission or diffusion of *Candida* spp. between dolphins and environments. However, there has been no investigation of the correlation between human pathogenic yeast isolates from dolphins and their keeping conditions, including captive pools and staff members, based on molecular biological studies.

The present study aims to investigate the fungal flora of pathogenic yeast species from the exhalation of dolphins, captive pools, staff members, and air in front of the dolphin show stage at the Churaumi Aquarium, Okinawa, Japan, to clarify correlations among the isolates from dolphins, captive environments and staff members using genotypes of multiple drug resistance 1 gene (*MDR1*) having adequate sites of diversity for strain identification [36] and the internal transcribed spacer (ITS) 1 5.8 s-ITS 2 regions of ribosomal RNA gene (*ITS rDNA*) sequences recommended as the bar-coding gene of pathogenic fungi for differentiation of species [37].

### 2. Materials and Methods

#### 2.1. Dolphins.

The fungal flora of exhalation in dolphins captive at the Churaumi Aquarium, Okinawa, Japan, were investigated. The investigations were carried out on 20 individuals in August 2006 as sample collections in the summer time, and February 2007 in the winter. The sampled dolphins were as follows: two bottlenose dolphins (*Trusios truncatus*), six (five in 2007 because of death) Indo-Pacific bottlenose dolphins (*Tursiops aduncus*), one Pacific bottlenose dolphin (*Trusios aduncus*), three dolphins of F1 offspring between bottlenose dolphins and Pacific bottlenose dolphins, two Pacific white-sided dolphins (*Lagenorynchus obliquiens*), six false killer whales (*Pseudorca crassidens*), and one rough-toothed dolphin (*Steno bredanensis*). The sex, age or estimated age, and housing periods in the aquarium are shown in Table 1. The survey was performed with the permission of Churaumi Aquarium, Okinawa, Japan, with a perspective for animal welfare.

#### 2.2. Isolation and Identification of Pathogenic Yeasts from Dolphins.

Four exhalations from each animal were collected. Two potato dextrose agar plates supplemented with 100 mg/L of chloramphenicol (CPDA) and two CHROMagar Candida plates (Kanto Chemical Co. Ltd., Tokyo, Japan) were placed approximately 40 cm above the blowhole. One plate per an exhalation was used. The plates were cultured at 25°C for 1 week, and the sprouted yeast colonies were counted. Colonies were collected according to slight differences in color on CHROMagar Candida plates and in size on CPDA. The collected yeast colonies were cultured on potato dextrose agar slants at 35°C for 48 hours. Colonies having growth ability at 35°C were identified on the basis of color on the CHROMagar Candida plates and species-specific polymerase chain reaction (PCR) for detecting topoisomerase II gene (*Top II PCR*) [43]. When plural isolates from one animal having identical genotype based on *MDR1* and *ITS rDNA* sequences have existed, the isolate was treated as one isolate. Mycelial colonies that grew on the plates were ignored at the present study.

#### 2.3. Captive Pools and Discharged Water to the Sea.

Samples were collected from eight pools of the aquarium for dolphins, two for manatees (*Trichechus manatus*), and at a discharging point of all pools to the sea. The relationship among pools and the water system is shown in Table 2.

Pools 1, 2, 3, and 8 communicated with each another and are supplied, seawater directly; this is indicated as water group A. Pools 4, 5, 6, and 7 indicated as water group B, also communicate with each another, and are supplied by sea water, and overflowing water from a fish aquarium. Pools 9 and 10 are supplied by salty well water, indicated as water group C. Seawater in discharge point is indicated as water D. Water for captive pools is taken 300 m from the shore and at a depth of 20 m. Water exchange by an overflow system works at 8, 4, and 24 times of the volume of water per day at water systems A, B, and C, respectively. A complete change of water is provided 2 or 3 times a week, and this is aided by a scrubbing brush and by using 12% concentration of hydrochloride solution.

Pools 1, 2, and 3 are used for the dolphin show at least 4 times a day, and sometimes the splash sprays on the audience. A special exhibition of training of a dolphin with an artificial tail fin is held at pools 4 and 5 at least twice a day, and the touching of dolphins by registered visitors is allowed every weekend and on holidays. Pools 9 and 10 also have opportunities for registered visitors to feed the manatees.

The inhabiting dolphin members are not fixed. They are placed depending on the programs of dolphin shows, health conditions, and affinities. In contrast, the housing of the manatees is fixed depending on the sex. The dolphins and manatees are nursed and treated by the same staff members. Foot-bathing tubs with hydrochloride are placed at each pool entrance.

#### 2.4. Isolation of Pathogenic Yeasts from Water Samples.

Five hundreds milliliter of water samples taken from the surface of the captive pools were filtered with a 0.22 μm pore-sized filter. The filters used for filtration were washed with 5 mL of sterile saline. One milliliter of the saline was put on CPDA and CHROMagar Candida plates and cultured at 25°C for 7 days. Yeast-form colonies were picked up and maintained on PDA slants at room temperature and identified by *Top II PCR* [43]. Mycelial colonies that grew on the plates were ignored at the present study.

#### 2.5. Oral Pathogenic Yeast Flora of Staff Members.

Twenty-four staff members (11 men, 13 women, 20–50 years old) were studied under personal agreement with informed consent. The survey was performed with the permission of Churaumi Aquarium, Okinawa, Japan. The ethic committee...
Table 1: Interpretive guidelines for in vitro susceptibility testing of Candida spp. extracted from CLSI guideline.

| Antifungal agent | Susceptible | Susceptible-dose dependent | Intermediate | Resistant | Nonsusceptible | Reference |
|------------------|-------------|---------------------------|--------------|-----------|----------------|-----------|
| Amphotericin B   | —           | —                         | —            | ≥1        | —              | [41]      |
| Flucytosine      | ≤4          | —                         | 8–16         | ≥32       | —              | [42]      |
| Fluconazole      | ≤8          | 16–32                     | ≥64          | ≥1        | —              | [42]      |
| Itraconazole     | ≤0.125      | 0.25–0.5                  | ≥2           | —         | —              | [42]      |
| Micafungin       | ≤2          | —                         | —            | —         | —              | —         |

∗: special term for echinocandin, and has as the same meaning as resistant.

Table 2: Dolphins and pathogenic yeasts isolates.

| No. | Dolphin Name | Animal species | Sex | Remarks age at Feb. 2007 | Aug. 2006 | Feb. 2007 |
|-----|--------------|----------------|-----|-------------------------|-----------|-----------|
| 1   | Gon          | FKW            | F   | Approx. 30 (Death record) | 16 C. tropicalis | 1 C. albicans |
| 2   | Sky          | BD             | M   | 7                        | 12 C. tropicalis | 59 C. tropicalis |
| 3   | Sami         | IOBD           | F   | 7                        | 9 C. albicans | 2 C. albicans |
| 4   | Fuji         | BD             | F   | Approx. 36                | 1 C. albicans | 1 C. albicans |
| 5   | Kana         | F1             | F   | 9 (died at 21, Aug. 2006) | 225 C. tropicalis | ND ND |
| 6   | Oki          | IOBD           | F   | Approx. 33                | 4 C. glabrata | 6 C. glabrata |
| 7   | Okigon-4     | FKW            | F   | Approx. 12                | 407 C. tropicalis | None — |
| 8   | Kama-2       | PWD            | M   | Unknown                   | None | None | |
| 9   | Cony         | F1             | F   | 17                       | 2 C. albicans | 23 C. albicans |
| 10  | Chao         | F1             | M   | 11                       | 601 C. tropicalis | 428 C. tropicalis |
| 11  | Kuro         | IOBD           | M   | Approx. 35                | 13 C. albicans | 2 C. albicans |
| 12  | Okigon-1     | FKW            | M   | Approx. 11                | None | None | |
| 13  | Larf         | RTD            | M   | Unknown (died at 3, Apr. 2008) | 2 C. albicans | 26 C. albicans |
| 14  | Okigon-3     | FKW            | F   | Approx. 35 (died at 15, Jan. 2008) | 291 C. tropicalis | 2425 C. tropicalis |
| 15  | Muku         | IOBD           | M   | Approx. 35                | None | None | |
| 16  | Dan          | IOBD           | M   | Approx. 38                | 20 C. albicans | None |
| 17  | Poi          | IOBD           | M   | Approx. 35                | 1 C. albicans | None |
| 18  | Chura        | FKW            | F   | 6 (died at 24, Dec. 2007) | None | None | |
| 19  | Momo         | FKW            | F   | Approx. 4                 | None | None | |
| 20  | Kama-1       | PWD            | F   | Unknown                   | None | None | |

FKW: False killer whale (Pseudorca crassidens), BD: Bottlenose dolphin (Tursiops truncatus), IOBD: Indo-Pacific bottlenose dolphin (Tursiops aduncus), PWD: Pacific white-sided dolphin (Lagenorhynchus obliquidens), F1: F1 offspring between BD and IOBD, RTD: Rough-toothed dolphin (Steno bredanensis), Approx.: Approximately, ∗: total numbers of colonies obtained from 2 CHROMagar Candida and 2 CPDA plates. Dolphin number 5 was died of enterocolitis and pneumonia, and numbers 13, 14 and 18 were of pneumonia caused by bacterial infections.

at the Chiba University judged that the present study had no infringement.

A sterile cotton tip was placed on the center of the tongue for 1 minute with rolling movements. The cotton tip was soaked in 2 mL of sterile-distilled water and then stirred vigorously for 10 seconds. One hundred microliters of the water was spread on a CHROMagar Candida plate cultured at 35°C for up to 7 days in duplicate. The sprouted yeast colonies were picked up and maintained on PDA slants at room temperature and identified by Top II PCR [43].

2.6 Airborne Fungi during Dolphin Show. One hundred liters and/or 500 L air was collected with an air sampler (Gunze, Tokyo, Japan) using an agar strip containing CPDA during the 4 dolphin shows at 11:00, 13:30, 15:00, and 16:00 in February 2007. The agar strips were cultured at 25°C for 7 days. The yeast colonies were picked up and cultured on PDA slants at 35°C for 2 and 7 days if the colonies were identified.

2.7 DNA Extraction. Fungal DNA was extracted with a DEXPAT Kit (TaKaRa, Ohtsu, Japan), following the manufacturer’s protocol with slight modification, from cultures incubated on PDA slants at 25°C for 48 to 96 hours. Approximately 100 µL of fungal mass was transferred to a sterilized microtube (1.5 mL) and homogenized with 0.5 mL of DEXPAT solution with a plastic pestle. The mixture was incubated at 100°C for 10 minutes and centrifuged at
12,000 pm (13, 201 g) for 10 min. The supernatant was used as the DNA sample.

2.8. Multiple Drug Resistant Gene 1 (MDR 1) Sequencing. The DNA was amplified with primers described by Tavanti et al. [36]. The primer sets for \textit{C. albicans} were MDR1_CAF \((5'\text{-TGT GTG TTA CCA TCT CT-3')}\) and MDR1_CAR \((5'\text{-AGG ACC AAA TAA TGG GA-3')}\), and those for \textit{C. tropicalis} were MDR1_CTF \((5'\text{-TGT TGG CAT TCA CCC TTC CT-3')}\) and MDR1_CTR \((5'\text{-TGG AGC ACC AAA CAA TGG GA-3')}\), DNA extract at 2.5 \(\mu\)L, a piece of Ready-to-Go beads (Amersham Pharmacia, Tokyo, Japan), 2.5 \(\mu\)L of 10 pM of the above primers, and 17.5 \(\mu\)L of distilled water were mixed. Amplification was performed for an initial denaturing step of 7 min at 94°C, 30 cycles of 1 min at 94°C for DNA denaturation, 90 seconds at 55°C for primer annealing, 90 sec at 72°C for primer extension, a final extension of 10 min at 72°C, and a 4°C soak. The amplified PCR product was confirmed by electrophoresis on 1.0% agarose in 1x/TBE buffer. Amplification was performed for an initial denaturing step of 7 min at 94°C, 30 cycles of 1 min at 94°C for DNA denaturation, 90 seconds at 55°C for primer annealing, 90 sec at 72°C for primer extension, a final extension of 10 min at 72°C, and a 4°C soak. The amplified PCR product was confirmed by electrophoresis on 1.0% agarose in 1x/TBE buffer. Amplification was performed for an initial denaturing step of 7 min at 94°C, 30 cycles of 1 min at 94°C for DNA denaturation, 90 seconds at 55°C for primer annealing, 90 sec at 72°C for primer extension, a final extension of 10 min at 72°C, and a 4°C soak. The amplified PCR product was confirmed by electrophoresis on 1.0% agarose in 1x/TBE buffer.

2.10. Genotypings. The MDR 1 and ITS rDNA sequences from both \textit{C. albicans} and \textit{C. tropicalis} were deposited in the GenBank via DDBJ (DNA database of Japan, Mishima, Shizuoka, Japan). The combined sequences of MDR 1 and ITS rDNA sequences were analyzed by the Unweighted Pair Group Method with Arithmetic mean (UPGMA) using GENETYX-MAC ver. 13.0 (GENETYX Corporation, Tokyo, Japan) genetic information processing software and given a serial number of genotype.

2.11. Susceptibility Testing. Susceptibility tests were performed according to the broth microdilution-modified method of the CLSI M27-A3 standard [41, 42] accepted standard using RPMI 1640 medium (Sigma, Poole, UK) buffered to pH 7.0 with MOPS (Sigma) and serial concentrations of amphotericin B (AMPH-B), flucytosine (5-FC), itraconazole (ITZ), flucanazole (FCLZ), micafungin (MCZ), and mica-fungin (MCFG). The latter three antifungals were included even though the method was originally described for use with AMPH-B, 5-FC, and ITZ. The test was performed in 96-well round-bottomed plastic plates using 100 mL of RPMI 1640 medium with fungal cells and antifungal substances. Data were obtained from duplicate trials. The mean or the lower data were taken. Reading results and the evaluation of the susceptibility categorized as susceptible, doze-dependent susceptible, intermediate, resistant and nonsusceptible on 5-FC, ITZ, FCLZ, and MCFG were followed to CLSI guideline [41, 42]. The category for susceptibility extracted from the CLSI guideline was shown in Table 1.

Twelve references isolates each for \textit{C. albicans} and \textit{C. tropicalis} stored in our center were added as references for molecular biological studies and the susceptibility test to antifungal substances.

3. Results

3.1. Isolates from Dolphins. The pathogenic yeasts isolated from exhalation of dolphins were \textit{C. albicans}, \textit{C. tropicalis}, and \textit{C. glabrata}. The total numbers of colonies and identified species are shown in Table 1. Fourteen out of 20 dolphins, corresponding to 70% of the animals, had some kinds of pathogenic yeast species. The holding rates of \textit{C. albicans}, \textit{C. tropicalis}, and \textit{C. glabrata} were 40%, 30%, and 5%, respectively.

Except for dolphin number 1, the rest of the fungal-positive animals had the same species of \textit{Candida} in investigations of both August 2006 and February 2007 (Table 2). The genotypes and susceptibility to antifungal drugs of the \textit{C. glabrata} isolate are not shown in the present study because there was only one isolate throughout the study.

Mycelial fungal species were also obtained from dolphin samples although the number of colonies was 1 or 2 per animal. The filamentous fungal species isolated from the exhalation of dolphins were shown in Table 3.

3.2. Isolates from Water, Staff Members, and Air. Collected water samples from 8 out of 11 sites from the captive pools and draining place had \textit{C. albicans} and/or \textit{C. tropicalis} during
the summer investigation while the winter investigation resulted in 4 of the 11 sites showing only C. albicans isolates. The total number of colonies was less than 5 regardless of the collecting time or place. Except for Pool No. 10 nursing the independent one. Isolates from dolphin No. 2 were identical regardless of the collecting time while dolphin No. 14 had different clones depending on the collecting time.

3.3. Consistent Fungal Species between Dolphins and Environments. The consistent fungal species through the dolphins, the water samples, and staff members were C. albicans and C. tropicalis, as shown in Tables 5 and 6, respectively. The plural isolates from an animal or a site at the same collection period were due to the size of the colony on CPDA and/or CHROMagar Candida, the color on CHROMagar Candida, and the genotype based on the combination of MDR1 and ITS rDNA.

3.4. MDR 1 and ITS rDNA Sequences in C. albicans. The sequences of MDR 1 in C. albicans, consisting of 645 bases, were divided into 16 genotypes with at least 98.8% identity, and those in ITS rDNA, consisting of at least 447 bases, were divided into 12 genotypes with at least 99.5% identity. Twenty-four genotypes based on combined sequences of MDR 1 and ITS rDNA among C. albicans isolates showed more than 99.2% identity. Although we tried to determine the ITS sequences on isolates IFM 55378, 55281, and 55298 many times, these sequences were impossible to complete, because of extremely overlapping sequence. The accession numbers of the genes and the genotypes were shown in Table 5.

Except for C. albicans isolates derived from the dolphin No. 11 having 2 different genotypes of C. albicans collected at the 2006 summer, there was no dolphin having different genotype simultaneously at the same collecting time. Candida albicans isolates from dolphin numbers 3, 4, and 13 showed different genotypes depending on the collecting seasons. Isolates IFM 55378 derived from the dolphin No. 9 and IFM 55281 from the dolphin No. 16 were treated as exceptions for genotyping analysis, because of lacking ITS rDNA sequences. Candida albicans isolates from the captive pools; numbers 1, 2, and 4 of the same collecting period showed different genotypes, except for the isolate IFM 55298 lacked the genotype of ITS rDNA.

The genotypes A, B, G, and H are common among C. albicans isolates from dolphins and environment. The genotype C is identical between a dolphin and a staff. There was no common genotype between the isolates from the aquarium and the references in C. albicans. There was no common genotype between the isolates from the aquarium and the references in C. albicans.

3.5. MDR 1 and ITS rDNA Sequences in C. tropicalis. The sequences of MDR 1 in C. tropicalis, consisting of 645 bases, were divided into 11 genotypes with at least 97.5% identity, and those in ITS rDNA, consisting of at least 435 bases, were divided into 5 genotypes with at least 94.5% identity. Thirteen genotypes based on combined sequences of MDR 1 and ITS rDNA among C. tropicalis isolates had more than 97.2% identity. In addition, the ITS rDNA sequence of isolate IFM 55379 derived from dolphin No. 10 was not determined and treated as an exception for genotype analysis because of extremely overlapping signals. The accession numbers of the genes and the genotypes were shown in Table 4.

Candida tropicalis isolates from dolphin No. 7 collected in the summer had 2 genotypes and in the winter had independent one. Isolates from dolphin No. 2 were identical irrespective of the collecting time while dolphin No. 14 had different clones depending on the collecting time.
The genotypes A and B are common among *C. tropicalis* isolates from dolphins and environment. In addition, the genotype A was detected not only in isolates at the aquarium but also in the reference ones.

### 3.6. Antifungal Susceptibility

The susceptibilities to antifungal agents were shown in Tables 5 and 6. No isolate showed resistance to AMPB among the *C. albicans* and *C. tropicalis* isolates from dolphins, environments, and reference.

Three of 15 (20%) from dolphins, 1 of 12 (8.3%) from the environments, and 1 of 12 (8.3%) from the references in *C. albicans* isolates showed resistance to 5-FC while none of *C. tropicalis* isolates regardless of origins showed resistance to 5-FC.

Thirteen of 15 (86.7%) from dolphins, 7 of 12 (58.3%) from the environments, and 1 of 3 (33.3%) from the staffs showed resistance or dose-dependent susceptibilities to FLCZ; however there was no isolate that showed resistance to the compound in the reference *C. albicans* isolates. Eight of 10 (80%) from dolphins, 1 of 6 (16.7%) from the environments, and 3 of 12 (25%) from the reference *C. tropicalis* isolates showed resistance or dose-dependent susceptibilities to FLCZ.

Twelve of 15 (80.0%) from dolphins, 10 of 12 (83.3%) from the environments, and 1 of 3 (33.3%) from the staffs showed resistance susceptibilities to ITZ; however no isolate showed resistance to the compound in the reference *C. albicans* isolates. Eight of 10 (80%) from dolphins, 3 of 6 (50%) from the environments, and 6 of 12 (50.0%) from the reference *C. tropicalis* isolates showed resistance or dose-dependent susceptibilities to ITZ.

There was no correlation between resistance or dose-dependent susceptibilities to antifungal agents and the genotype of *MDR1* or *ITS rDNA* in either *C. albicans* or *C. tropicalis* isolates. In addition, one isolate derived from the captive pool no. 5 collected at the winter 2007 showed extremely resistant to MCZF as 16 µg/mL. The susceptibilities to MCZ were listed as reference data.

### 4. Discussion

#### 4.1. Isolating Rates for Pathogenic Yeasts from Dolphins

According to Buck et al., the holding rates of *Candida* spp. in free-ranging dolphins were as follows: *C. albicans*, 7.0%; *C. tropicalis*, 14.3%; and *Candida* sp., 14.3% [35]. At the present study, the holding rates of *Candida* spp. in dolphins and captive-pools were 70% and 90.9%, respectively, which were higher than those of free-ranging dolphins. Similarly, another report by Buck et al. [35] demonstrated that the captive environments of dolphins showed a higher incidence, over 70%, in feces and pool waters of captive bottlenosed dolphins (*Tursiops truncatus*). It suggested that the data from various aquariums or institutions might vary depending on the nursing conditions and the climates of the aquarium. Further studies will confirm the average data of the holding ratio of pathogenic yeast species in captive dolphins and their nursing environments with considerations of age, sex, and physiological data.

#### 4.2. Relationship between Fungal Exhalation and Health

The relationship between fungal exhalation phenomena from blowholes and health condition has not been confirmed [3, 19], although many veterinarians, animal-keepers, and nurses in aquariums in Japan consider the isolations of *Candida* spp. from exhalation as being indicative of illness or weakness in dolphins [34]. We agree that a small numbers
| IFM No. | Animal No. | AMPH-B | 5-FC | FLCZ | ITZ | MCZ | MCFG | MDR 1 | ITSrRNA | Combined |
|---------|------------|--------|------|------|----|----|------|-------|--------|----------|
| 55372   | No. 1 (W)  | 0.5    | <0.125 | >64* | >8* | 4  | <0.03 | AB379716 | 1      | AB437006 | V A      |
| 55224   | No. 3 (S)  | 0.5    | <0.125 | >64* | >8* | 8  | <0.03 | AB379697 | 1      | AB436989 | VI B     |
| 55374   | No. 3 (W)  | 0.5    | <0.125 | >64* | >8* | 2  | <0.03 | AB379717 | 1      | AB437007 | VI B     |
| 55226   | No. 4 (S)  | 0.25   | 0.25  | 32*  | 8*  | 2  | <0.03 | AB379699 | 16     | AB436991 | V C      |
| 55376   | No. 4 (W)  | 0.25   | 0.125 | 1    | 0.125 | 2 | <0.03 | AB379718 | 16     | AB437008 | V C      |
| 55292   | No. 5 (S)  | 0.5    | <0.125 | 64*  | 8*  | 2  | <0.03 | AB379706 | 1      | AB436997 | VI B     |
| 55267   | No. 9 (S)  | 0.25   | >64*  | >64* | >8* | 1  | <0.03 | AB379700 | 15     | AB436992 | VI D     |
| 55378   | No. 9 (W)  | 0.25   | >64*  | >64* | >8* | 32 | <0.03 | AB379719 | 15     | ND ND ND | ND       |
| 55273   | No. 11 (S) | 0.25   | 0.125 | >64* | >8* | 4  | <0.03 | AB379701 | 15     | AB436993 | VI D     |
| 55274   | No. 11 (S) | 0.5    | <0.125 | 2    | 0.125 | 1 | <0.03 | AB379702 | 15     | AB436994 | V E      |
| 55381   | No. 11 (W) | 0.25   | <0.125 | 64*  | 2*  | 8  | <0.03 | AB379720 | 15     | AB737009 | XI F     |
| 55276   | No. 13 (S) | 0.25   | <0.125 | >64* | >8* | 4  | <0.03 | AB379703 | 7      | AB436995 | V G      |
| 55382   | No. 13 (W) | 0.25   | <0.125 | 64*  | 8*  | 4  | <0.03 | AB379721 | 7      | AB437010 | V G      |
| 55281   | No. 16 (S) | 0.125  | >64*  | >64* | >8* | 2  | <0.03 | AB379704 | 15     | ND ND ND | ND       |
| 55290   | No. 17 (S) | 0.5    | <0.125 | 8    | 0.125 | <0.06 | <0.03 | AB379705 | 1      | AB436996 | IX H     |
| 55384   | Pool-No. 1 (A) (W) | 0.5 | <0.125 | >64* | >8* | 2  | <0.03 | AB379722 | 1      | AB437011 | VI B     |
| 55385   | Pool-No. 1 (A) (W) | 0.5 | <0.125 | 0.125 | 0.03 | 0.06 | <0.03 | AB379723 | 7      | AB437012 | V G      |
| 55302   | Pool-No. 2 (A) (S) | 0.5 | <0.125 | >64* | >8* | 2  | <0.03 | AB379710 | 1      | AB737000 | IX H     |
| 55304   | Pool-No. 2 (A) (S) | 0.25 | <0.125 | 64*  | >8* | 2  | <0.03 | AB379711 | 1      | AB437001 | VI B     |
| 55298   | Pool-No. 3 (A) (S) | 0.5 | <0.125 | >64* | >8* | 2  | <0.03 | AB379709 | 1      | ND ND ND | ND       |
| 55295   | Pool-No. 4 (B) (S) | 0.5 | <0.125 | 4    | >8* | 2  | <0.03 | AB379707 | 1      | AB436998 | VI B     |
| 55867   | Pool-No. 4 (B) (S) | 0.5 | <0.125 | 4    | 2*  | 2  | <0.03 | AB379708 | 1      | AB436999 | IX H     |
| 55388   | Pool-No. 5 (B) (W) | 0.25 | <0.125 | >64* | >8* | 16 | >16 | AB379726 | 1      | AB437015 | V A      |
| 55387   | Pool-No. 7 (B) (W) | 0.25 | >64*  | >64* | >8* | >32 | <0.03 | AB379725 | 15     | AB437014 | VIII I   |
| 55386   | Pool-No. 8 (A) (W) | 0.25 | <0.125 | >64* | >8* | >32 | <0.03 | AB379724 | 1      | AB437013 | VIII J   |
| 55871   | Pool-No. 9 (C) (S) | 0.5 | <0.125 | 8    | 1*  | 2  | <0.03 | AB379712 | 15     | AB707002 | VIII I   |
| 55319   | Sea Water (D) (S) | 0.25 | <0.125 | 4    | 0.06 | 2  | <0.03 | AB379714 | 15     | AB437004 | VIII I   |
| 55390   | Staff-A     | 0.25 | <0.125 | >64* | >8* | 8  | <0.03 | AB379727 | 16     | AB437016 | V C      |
| 55392   | Staff-B     | 0.5   | <0.125 | 2    | 0.125 | 0.5 | <0.03 | AB379728 | 11     | AB437017 | V K      |
| 55395   | Staff-C     | 0.5   | <0.125 | 0.5  | 0.03 | 2  | <0.03 | AB379729 | 5      | AB437018 | II L     |
| 4953    | Sputum (J)  | 0.25 | <0.125 | 0.25 | 0.03 | <0.06 | <0.03 | AB379730 | 9      | AB437019 | I M      |
| 5633    | Oral mucosa (J) | 0.25 | <0.125 | 0.25 | 0.03 | <0.06 | <0.03 | AB379731 | 13     | AB437020 | V N      |
| 5713    | Sputum (J)  | 0.25 | <0.125 | 0.125 | 0.03 | <0.06 | <0.03 | AB379732 | 10     | AB437021 | IV O     |
| 40213   | Blood (USA) | 0.5   | 0.25  | 0.25 | 0.03 | 0.06 | <0.03 | AB379735 | 2      | AB437024 | V P      |

Table 5: Antifungal susceptibility and genotypes of C. albicans isolates.
of total colonies in *C. albicans*, *C. glabrata*, and *C. tropicalis* isolates might be attached as normal fungal residents of mucous membranes, as described by Buck in 1980 [19], however, we had a doubt on the negative correlation between large numbers of *Candida* spp. colonies and a predictive sign of weakened health or preillness. There were 4 dolphins that dead after August 2006; for example dolphin No. 5 died in August 21, 2006 by pneumonia and colitis, No. 18 in December 24, 2007, No. 14 in January 15, 2008, and No. 13 in April 3, 2008 by pneumonia with long-term treatments. Two out of 4 dolphins showed a large numbers of *Candida* spp. in the breath when sampled indicating the correlation between large numbers of *Candida* spp. colonies and a predictive sign of weakened health or preillness. On the other hand, dolphin No. 7 had 407 *C. tropicalis* colonies in the summer and none in the winter, dolphin No. 10 had 601 colonies in the summer and 428 colonies in the winter did not die, and the majority of live dolphins had low *Candida* spp. rates. We could not confirm the relationship between the number of *C. tropicalis* colonies and the health condition of dolphins from these findings.

Although some pathogenic mycelial fungal species such as *Aspergillus niger*, *Aspergillus sp.*, *Phoma sp.*, *Curvularia lunata*, *Aspergillus sp.*, *Schizophyllum commune*, *Aureobasidium pullulans*, and *Fusarium* sp. were isolated from exhalation from blowholes, there was no correlation on the health of dolphins. We should wait for the accumulation of data on the fungal flora from exhalation and body conditions including blood and other physiological examinations, for judging the existence of the correlation.

4.3. Seasonal Characteristics on the Isolates. Interestingly, water source-derived *C. tropicalis* isolates disappeared in the winter, suggesting that *C. tropicalis* might have some difficulty surviving in winter conditions, and even in subtropical areas. The average temperatures of the environment and captive pools in February were 19.2°C and 22.2°C, while those in August were 31.4°C and 28.8°C, respectively. It is considered that the differences in water temperature might be one of the factors for the existence of *C. tropicalis*. Future investigations may confirm this phenomenon.

### Table 5: Continued.

| IFM No. | Animal No. | AMPH-B | 5-FC | FLCZ | ITZ | MCZ | MCFG | MDR 1 Accession No. | Genotype (I–X) | ITS rDNA Accession No. | Genotype (I–XII) | Combined Genotype (A–X) |
|---------|------------|--------|------|------|-----|-----|-----|-------------------|----------------|----------------------|------------------|-----------------------|
| 41419   | Sputum (J) | 0.25   | <0.125 | 0.25 | 0.03 | 0.06 | <0.03 | AB379736          | 4               | AB437025             | X                 | Q                     |
| 49764   | Oral mucosa (J) | 0.5 | <0.125 | 0.25 | 0.03 | 0.06 | <0.03 | AB379741          | 4               | AB437029             | V                 | R                     |
| 49765   | Oral mucosa (J) | 0.5 | >64* | 8    | 0.06 | 0.06 | <0.03 | AB379742          | 3               | AB437030             | III               | S                     |
| 49767   | Tongue (J) | 0.5 | <0.125 | 1    | 0.06 | 0.25 | <0.03 | AB379743          | 6               | AB437034             | XII               | T                     |
| 54349   | Sputum (J) | 0.5 | <0.125 | 0.25 | 0.03 | 0.06 | <0.03 | AB379751          | 8               | AB437039             | I                 | U                     |
| 54381   | Sputum (J) | 0.5 | <0.125 | 0.125 | 0.03 | 0.125 | <0.03 | AB379752          | 14              | AB437040             | V                 | V                     |
| 54604   | Clinical isolate (TW) | 0.5 | | 1    | 1    | 0.125 | 0.25 | <0.03 | AB379754          | 16              | AB437041             | XII               | W                     |
| 55046   | Clinical isolate (J) | 0.5 | <0.125 | 0.125 | 0.03 | 0.06 | <0.03 | AB379756          | 12              | AB437043             | VII              | X                     |

S: collected at August 2006, W: collected at February 2007.
J: Japan, USA: the United States of America, TW: Taiwan.
AMPH-B: amphotericin B, 5-FC: flucytosine, FLCZ: fluconazole, ITZ: itraconazole, MCZ: miconazole, MCFG: micafungin:
*: resistant, and +; dose-dependent susceptibility based on CLSI M27-A2 protocol [42].

### 4.4. Genotypes. Genotypes based on MDR 1 seemed to be suitable for molecular epidemiological study in a confined area due to adequate sites of diversity [36, 39]. On the other hand, genotypes of ITS rDNA could be useful for the identification of some intraspecies diversity and strain differentiations [44], and could show correlations to geographic, regional, and/or host-dependent genotypes of pathogenic fungi determined by multiple gene analysis [45]. Furthermore, the combination of 2 genes; MDR 1 and ITS rDNA, could indicate more detailed diversity of the genotypes of *Candida* spp. than those by MDR 1 or ITS rDNA alone. Then we discussed on the distributions of genotypes for *C. albicans* and *C. tropicalis* isolated in the aquarium based on the combined genotypes of MDR 1 and ITS rDNA. In the basis of these combinations, we could demonstrate the existences of coincident pathogenic yeast species and their genotypes in both *C. albicans* and *C. tropicalis* between or among dolphins, captive-pools and a staff member although Buck have denied the possibility that pathogenic yeasts are dispersed to other dolphins and environments [19]. Especially, the common genotype of *C. albicans* to both a dolphin and a staff member might be exchanged between them since the animal has been receiving medicine and surgical treatments from the staff member working as a veterinarian.

Interestingly, the genotype of *C. albicans* isolated from dolphin No. 5 which suddenly died of bacterial colitis and pneumonia was detected in the isolate from dolphin No. 3.
and captive pools numbers 1, 2, and 4. Those of *C. tropicalis*
isolated from the same dolphin No. 5 have also been detected
in the isolate from dolphin No. 10, from captive pools No. 5
and No. 6, and from drained seawater. The animal might be a
dispersal source for both *C. albicans* and *C. tropicalis* to other
animals as well as to the environment.

The source of pathogenic yeasts might be related to the
environmental water since common genotypes among the
dolphins and pool water samples were found in *C. albicans*
and *C. tropicalis* isolates. Isolates from captive pools free of
dolphins had the same genotypes of *Candida* spp. isolates
as the dolphins. Nevertheless, any systematic relationship of
the water supply could not be found between pathogenic
yeast species and captive pools or seawater. Exchanges of
the water supply could not be found between pathogenic
animal swells to other environments.

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### Table 6: Antifungal susceptibility and genotypes of *C. tropicalis* isolates.

| IFM No. | Animal No. | Genotype | Accession No. | Combined Genotype |
|---------|------------|----------|---------------|------------------|
| Dolphin isolates | | | | |
| 55217 | No. 1 (S) | <0.125 | AB37975 | |
| 55220 | No. 2 (S) | <0.125 | AB37979 | |
| 55373 | No. 3 (W) | <0.125 | AB37977 | |
| 55229 | No. 4 (S) | <0.125 | AB37976 | |
| 55233 | No. 5 (S) | <0.125 | AB37976 | |
| 55329 | No. 6 (S) | <0.125 | AB37976 | |
| 55269 | No. 7 (S) | <0.125 | AB37976 | |
| 55379 | No. 8 (W) | <0.125 | AB37978 | |
| 55377 | No. 9 (S) | <0.125 | AB37976 | |
| 55383 | No. 10 (W) | <0.125 | AB37978 | |

### Environmental isolates

| IFM No. | Animal No. | Genotype | Accession No. | Combined Genotype |
|---------|------------|----------|---------------|------------------|
| 55294 | Pool-No. 1 (A) (S) | <0.125 | AB37977 | |
| 55297 | Pool-No. 2 (A) (S) | <0.125 | AB37977 | |
| 55299 | Pool-No. 3 (A) (S) | <0.125 | AB37977 | |
| 55301 | Pool-No. 4 (B) (S) | <0.125 | AB37977 | |
| 55303 | Pool-No. 5 (B) (S) | <0.125 | AB37977 | |
| 55318 | Sea Water (D) (S) | <0.125 | AB37977 | |

### Reference isolates

| IFM No. | Animal No. | Genotype | Accession No. | Combined Genotype |
|---------|------------|----------|---------------|------------------|
| 5476 | Clinical isolate (J) | <0.125 | AB37978 | |
| 41420 | Clinical isolate (J) | <0.125 | AB37978 | |
| 52008 | Clinical isolate (J) | <0.125 | AB37978 | |
| 52101 | Clinical isolate (J) | <0.125 | AB37978 | |
| 52103 | Clinical isolate (J) | <0.125 | AB37979 | |
| 52938 | Clinical isolate (J) | <0.125 | AB37979 | |
| 53910 | Clinical isolate (J) | <0.125 | AB37979 | |
| 54637 | Clinical isolate (J) | <0.125 | AB37979 | |
| 54674 | Clinical isolate (J) | <0.125 | AB37979 | |
| 55049 | Clinical isolate (J) | <0.125 | AB37980 | |
| 55256 | Clinical isolate (J) | <0.125 | AB37980 | |

S: collected at August 2006, W: collected at February 2007, J: Japan.
A, B, C and D indicated at the environmental isolate indicated the water supply system.
AMPH-B: amphotericin B, 5-FC: flucytosine, FLCZ: fluconazole, ITZ: itraconazole, MCZ: miconazole, MCFG: micafungin.
*: resistant, and +: dose-dependent susceptibility based on CLSI M27-A2 protocol [42].
The coincidence of genotypes in dolphins and in environmental isolates to the reference isolates of *C. tropicalis* suggested that such genotypes might be introduced from audiences or from sea water, and/or be very common in the world.

4.5. **Attention for Sample Collection.** Attention to plural isolates from the same animal at the same sampling period might be important. Although the differences in the colonies were slight with regard to size on CPDA or color on CHROMagar Candida, the clones showed different genotypes and/or susceptibilities to antifungal agents as detected in the *C. albicans* isolates IFM 55273 and IFM 55274 derived from dolphin No.11 isolated at the August 2006. Therefore, at least 2 or more colonies, depending on size and/or color, should be selected for identification, molecular biological analysis, and susceptibilities to antifungal drugs.

4.6. **Risk to Be Audience.** The fishy smell in the auditorium of the dolphin show indicates a possible spread of the breath, including pathogenic yeasts, to the audience, in spite of the fact that such pathogenic yeast isolates from air samples collected in the front of the dolphin shows were not detected. This suggests that the possibility of inhaling or being exposed to pathogenic *Candida* spp. from the exhalation of dolphins is relatively low. Nevertheless, it seems dangerous to approach the blowholes to a distance closer than 40 cm. For example, activities such as kissing or touching dolphins and, for pregnant women, swimming with dolphins should be approached with caution. The exact distance from the blowholes of dolphins from where it would be free of yeast-blow needs to be measured. Although there was no record of fungal infection caused by inhalation of the exhalation of dolphins, an immunocompromised person should be strongly urged to avoid such close contact with dolphins.

4.7. **Characteristics on the Susceptibilities to Antifungal Drugs.** The high ratio in isolations of pathogenic yeasts derived from the dolphins compared with the reference strains was the same as that in human oral fungal flora with HIV-infected or immunocompromised hosts [46, 47]. Frequent administrations of antibiotics and steroids might be one of the explanations, but data regarding the parameters concerning stress and immunosuppression, defense mechanisms against microorganisms, and drug metabolisms in the animals, even in normal immune data or blood chemical profiles, are not yet sufficient for meaningful discussion, although the correlation between the occurrence of lobomycosis and immune status of the dolphins was reported in Floridan bottlenose dolphins [10, 48–51]. Furthermore, the higher incidences of resistance to azole-related antifungal agents in the isolates from dolphins and environments might be related to the sodium chloride in sea water, a speculation drawn from the correlations among resistance to chemical compounds, pathogenicity, and sodium chloride [52].

4.8. **Correlation between the Genotype and Resistance to Antifungal Agents.** According to Tavanti et al., MDR 1 alone cannot define the relationship between genotypes and profiles of antifungal agents [36]. In the present study, no correlation was found between resistance or dose-dependents susceptibilities to azole-related antifungal agents and the genotypes of *MDR1* and/or *ITS rDNA* in either *C. albicans* or *C. tropicalis*, since an identical genotype based on *MDR1* and *ITS rDNA* sequences has shown different profiles of susceptibilities to azole-related antifungal agents, regardless of origin. The reference isolates, giving a higher ratio of resistant isolates, could not help in determining the specific genotypes based on the combination of *MDR1* and *ITS rDNA* sequences.

5. Conclusions

The detection of common genotypes on *Candida* spp. among dolphins, environments, and staff members pointed to the dissemination of pathogens at the aquarium. Thus, it seemed to be important to consider the effects on audiences from dolphins and the reverse relations for controls of zoonotic infections. In addition, the sequence of *MDR 1* showed adequate numbers of variations, indicating that the gene might be useful for molecular epidemiological studies.

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