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NOTE

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Isolation of antifungal-resistant *Candida* from the blowholes of captive dolphins

Chika SHIRAKATA[1)], Sakura SUGAWARA[2)] and Rui KANO[2,3)]*

[1)]Enoshima Aquarium, Kanagawa, Japan
[2)]Department of Veterinary Dermatology, Nihon University College of Bioresource Sciences, Kanagawa, Japan
[3)]Present address: Teikyo University Institute of Medical Mycology (TIMM), Tokyo, Japan

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ABSTRACT. In this study, we isolated eight strains of *Candida albicans* from the blowhole air cultures of eight dolphins (one Pacific white-sided dolphin and seven bottlenose dolphins) housed at the Enoshima Aquarium. The minimum inhibitory concentrations of antifungals for these isolates were determined by conducting E-test and broth microdilution assays using the CLSI M27-A3 protocol antifungal susceptibility testing method. Only one of the eight dolphins from which *Candida* had been isolated had been treated with amphotericin B (AMB), and four had been treated with itraconazole (ITZ). All isolates were identified as *Candida albicans*, and all were resistant to both ITZ and voriconazole, though the isolates exhibited susceptibility to AMB and micafungin. Based on our findings, we suspect that the frequency of occurrence of azole-resistant *Candida* species is increasing in captive dolphins as well as in their aquarium environments.

KEYWORDS: antifungal-resistance, *Candida albicans*, dolphin, susceptibility testing

Pulmonary mycosis is a fungal infection that frequently is caused by *Candida* spp. and commonly affects dolphins [4, 10]. Various antifungal compounds, such as flucytosine, fluconazole, itraconazole (ITZ), voriconazole (VRZ), amphotericin B (AMB), and micafungin (MCF), are used to treat respiratory fungal infections in dolphins [4, 7, 10]. These compounds also have been used for severe candidiasis in humans, but recently, clinical strains resistant to these drugs have become a problem in Japan [11]. Drug-resistant candidiasis makes treatment difficult and increases mortality in human patients [11]. Therefore, drug-resistant candidiasis in dolphins may cause similar problems. Moreover, joint monitoring of antifungal drug-resistant fungi in humans, animals, and the environment is an important part of the One Health approach to controlling the emergence and spread of antimicrobial resistances [2].

In a previous study, we isolated ITZ- and VRZ-resistant *Candida albicans, C. glabrata*, and *C. tropicalis* from blowhole air cultures of dolphins housed at the Port of Nagoya Public Aquarium, Japan [8]. It was not clear whether the isolation of these resistant strains was specific to the dolphins at the Port of Nagoya Public Aquarium or would be found in other aquaria in Japan.

In the present study, we isolated *Candida* species from the blowhole air cultures of eight dolphins housed at a distinct site, the Enoshima Aquarium and determined the minimum inhibitory concentrations (MICs) of antifungals on these isolates by conducting antifungal susceptibility testing.

A total of twelve captive dolphins (two Pacific white-sided dolphin [*Lagenorhynchus obliquidens*], one Risso's dolphin [*Grampus griseus*] and nine bottlenose dolphins [*Tursiops truncatus*]) in two different pools were examined in this study. Notably, Pool 1 (holding nine bottlenose dolphins and one Risso's dolphin) and Pool 2 (holding two Pacific white-sided dolphins) were completely independent. Water for these pools consisted of natural sea water drawn from an intake 200 m offshore, using a suction line that is buried 2 m under the sea floor. Both Pool 1 and Pool 2 are equipped with individual closed-loop water circulation systems (FU-I [custom-made], Ebara-Corp., Tokyo, Japan) individually. These water circulation systems are capable of circulating water in each pool 12 times per day, providing complete replacement of each pool's entire water volume approximately every 10 days. Both pools are disinfected by adding sodium hypochlorite to achieve a residual chlorine concentration of 0.2–0.5 mg/l. Information on the dolphins and antifungal medications is shown in Table 1. No dolphins showed clinical signs (malodorous breath, fever, or sputum discharge) at the time of sampling. One dolphin had been treated with AMB (2 mg/kg) orally three times per day for 48 days in response to *Candida* gastritis (Table 1).

A total of eight strains of *C. albicans* (one from each sample animal) were collected from the blowholes of the eight dolphins on 5th November 2021; each sample was collected directly onto CHROMagar™ Candida medium (Kanto Chemical Co., Inc., Tokyo, Japan) placed approximately 30 cm above the blowhole to receive a voluntary exhalation that was a part of the animals'

*Correspondence to: Kano, R.: kanou.rui.ng@teikyo-u.ac.jp, Teikyo University Institute of Medical Mycology (TIMM), 359 Otsuka, Hachioji, Tokyo 192-0395, Japan

husbandry training.

Following use in sampling, plates were incubated at 37°C for 48 hr. All yeast colonies that grew on each plate were green and had a smooth surface. Yeast colonies were identified as *Candida* species based on the sequence homology of the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2), as described previously [13]. The universal fungal primers, ITS-5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′), were used to amplify the ITS region of the isolates [13]. Comparative nucleotide sequence analysis using the BLAST algorithm on the NCBI website showed that the ITS sequences amplified from each of the isolates were 100% identical to those of the *C. albicans* CBS 2732 ITS region (GenBank Accession No. KY101911). Therefore, all strains were identified as *C. albicans* based on ITS region sequence analysis (Table 2).

Table 1. Information on dolphins and antifungal medications with strain number

Dolphin number	Sex	Species	Age (years)	Date of acquisition	Pool No.	Present medication for antifungal drug	History of antifungal treatments	Strain number
1	Female	Bottlenose dolphin (*Tursiops truncatus*)	9	Jun. 2012	1	AMB, 2 mg/kg, PO, TID, for 48 days	ITZ, 2.5 mg/kg, PO, BID, for 30 days (63 months ago), for 47 days (44 months ago), for 14 days (32 months ago), for 28 days (22 months ago). AMB, 2 mg/kg, PO, TID, for 21 days (43 months ago, 33 month ago, 21 month ago).	NUBS21-1
2	Male	Pacific white-sided dolphin (*Lagenorhynchus obliquidens*)	E[1)] 44	Feb. 1978	2	NO	ITZ, 2.5 mg/kg, PO, BID, for 22 days (57 months ago).	NUBS21-2
3	Female	Bottlenose dolphin (*T. truncatus*)	21	Jun. 2000	1	NO	ITZ, 2.5 mg/kg, PO, BID, for 20 days (88 months ago).	NUBS21-3
4	Female	Bottlenose dolphin (*T. truncatus*)	E 29	Dec. 1994	1	NO		NUBS21-4
5	Female	Bottlenose dolphin (*T. truncatus*)	E 29	Oct. 1996	1	NO		NUBS21-5
6	Female	Bottlenose dolphin (*T. truncatus*)	E 45	May. 1988	1	NO	ITZ, 2.5 mg/kg, PO, BID, for 14 days (71 months ago).	NUBS21-6
7	Female	Bottlenose dolphin (*T. truncatus*)	E 6	Dec. 2018	1	NO		NUBS21-7
8	Female	Bottlenose dolphin (*T. truncatus*)	19	Jul. 2002	1	NO		NUBS21-8
9	Male	Pacific white-sided dolphin (*L. obliquidens*)	E 24	Dec. 2005	2	NO		-
10	Female	Risso's dolphin (*Grampus griseus*)	E 35	Jan. 1988	1	NO	ITZ, 2.5 mg/kg, PO, BID, for 14 days (43 months ago).	-
11	Female	Bottlenose dolphin (*T. truncatus*)	E 5	Dec. 2018	1	NO		-
12	Male	Bottlenose dolphin (*T. truncatus*)	1.6	Apr. 2020	1	NO		-

E: estimated; AMB: amphotericin B; ITZ: itraconazole; PO: per os; TID: ter in die; BID: bis in die.

Table 2. Antifungal susceptibilities of eight isolates

Strain number	Species	Status of colonies	No. of colonies on the plate	MICs (μg/ml)				
				AMB	FLZ	ITZ	VRZ	MCF
NUBS21-1	*Candida albicans*	Green, smooth	183	0.5	>256	>32	>32	0.004
NUBS21-2	*C. albicans*	Green, smooth	14	0.003	>256	2	>32	0.008
NUBS21-3	*C. albicans*	Green, smooth	>1,000	0.25	>256	>32	>32	<0.002
NUBS21-4	*C. albicans*	Green, smooth	>1,000	0.19	>256	>32	>32	0.002
NUBS21-5	*C. albicans*	Green, smooth	408	0.25	>256	>32	>32	0.008
NUBS21-6	*C. albicans*	Green, smooth	>1,000	0.125	>256	>32	>32	<0.002
NUBS21-7	*C. albicans*	Green, smooth	240	0.5	>256	>32	>32	0.002
NUBS21-8	*C. albicans*	Green, smooth	210	0.25	>256	>32	>32	0.008

MICs: minimum inhibitory concentrations; AMB: amphotericin B; FLZ: fluconazole; ITZ: itraconazole; VRZ: voriconazole; MCF: micafungin.

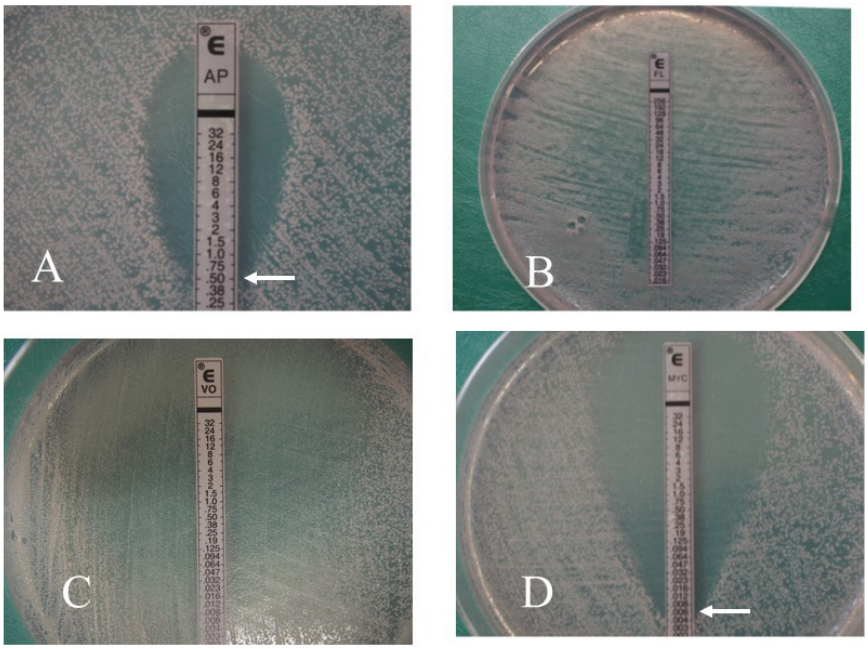

Fig. 1. E-test being used to determine the minimum inhibitory concentrations (MICs) of strain NUBS21-1 to amphotericin B (AMB) (**A**), fluconazole (FLZ) (**B**), voriconazole (VRZ) (**C**) and micafungin (MCF) (**D**: the E-test shows micafungin abbreviated as MYC). Arrows indicate MIC (μg/ml) of each drug. On the cases of FLZ and VRZ strips (B and C), colony growths were not inhibited, so the MICs were determined to be >256 μg/ml and >32 μg/ml, respectively.

To performe to determine the MICs of AMB, MCF, and VRZ for the eight isolates of *Candida* collected from the blowholes of the eight dolphins; E-tests (bioMérieux Japan, Tokyo, Japan) were performed as described in the E-test Technical Guide (https://www.ilexmedical.com/files/E-test-Package-Insert/AntifungalSusceptibilityTesting.pdf). The minimum inhibitory concentration (MIC) of azoles was defined for showing 80% growth inhibition as judged by the naked eye.

In addition, given discontinuation of the ITZ E-test strips, the MICs of ITZ were determined by the broth microdilution assay of the Clinical Laboratory Standards Institute (CLSI) M27-A3 protocol [3]. For quality control, the strain *Candida parapsilosis* ATCC 22019 was used in CLSI M27-A3 to check the accuracy of drug dilution [3]. All MIC determination experiments were performed in duplicate.

Candida spp. were classified as susceptible (S), susceptible dose-dependent (S-DD), or resistant (R) to ITZ and VRZ according to the clinical breakpoints in the M27-A3 guidelines as provided by the CLSI [1]. The MICs of ITZ for S, S-DD, and R isolates were defined as ≤0.125 μg/ml, 0.25–0.5 μg/ml, and ≥1 μg/ml, respectively. The MICs of VRZ on S and R isolates were defined as ≤1 μg/ml and ≥4 μg/ml, respectively [1]. All isolates of *C. albicans* obtained in the present study were R to both ITZ and VRZ, and S to AMB and MCF (Fig. 1; Table 2).

It is important to understand the origin of drug-resistant microbes in wildlife as part of One Health initiatives. In aquaria, fungi have been detected in the environment of breeding facilities, such as air samples and walls, and in care staff [5, 13]. Drug-resistant microbes also may be transferred via close contact between humans and animals [9]. Schmid *et al.* previously warned that the higher incidences of resistance to azole related antifungal agents in the isolates from dolphins and associated environments may be related to the level of sodium chloride in sea water [12]. In the future work, we hope to investigate possible associations between seawater and antifungal drug resistance in *C. albicans*.

Our previous study was the first (to our knowledge) to report VRZ-resistant *C. albicans* and *C. tropicalis* isolates from the blowholes of dolphins, as assessed at the Port of Nagoya Public Aquarium of Japan [8]. In the present study, we also isolated multi-azole-resistant *C. albicans* from dolphins that did not have a history of antifungal treatment. Dolphin 1, as well as other dolphins had a history of ITZ treatment over the preceding eight years, suggesting that resistance to azole agents may have resulted from exposure to ITZ within the aquarium environment (Table 1). In addition, as Takahashi *et al*. [13] have noted azole-resistant *Candida* spp. are transmissible between captive dolphins through droplet infection or contaminated pool water, routes that also may apply in the present study. In the present study, Pool 1 and Pool 2 at the Enoshima Aquarium were maintained via independent water pumping systems, but the acquisition emergence of azole-resistant *Candida* was observed across both pools. Based on these results, we hypothesize that the presence of azole-resistant *Candida* in the dolphins examined in this study may have originated from human sources, such as care staff or visitors, and that these microbes may have spread through care staff or the environment. To reduce the transmission of resistant fungi to dolphins and contamination of the environment, it is important to take measures to prevent humans from introducing the fungi and to disinfect the environment to prevent transmission between individuals. In the present study, only one species, *C. albicans*, was detected, but this may be because *C. tropicalis* and *C. glabrata* did not enter the

facility and then spread among the captive dolphins within the aquarium environment.

Drug susceptibility testing is not yet common in Japanese aquaria [6]. Azoles are the most used antifungal drugs in dolphins [10]. Thus, it is important to conduct culture and susceptibility testing before administering medication and to select appropriate antifungal agents other than azoles when treating for azole-resistant *Candida* spp. infections in dolphins. In addition, it is advisable to conduct drug susceptibility screening of *Candida* spp. as a routine test to keep update on the status of azole resistance. If an outbreak of azole-resistant fungi is confirmed in an aquarium, the use of antifungal agents other than azole antifungals should be considered as a first choice.

Based on our findings, we suspect that the occurrences of azole-resistant *Candida* species are increasing in dolphins and their aquarium environments. These azole-resistant *Candida* species might directly or indirectly infect humans, but are not thought to be pathogenic in healthy humans. Nonetheless, the control of drug-resistant *Candida* is necessary for both dolphin and human health.

This study did not require approval from an animal welfare and ethics committee, given that this work represented health management for fungal infection in captive dolphins, and that sampling was performed by a non-invasive method.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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