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First isolation of voriconazole-resistant *Candida albicans*, *C. tropicalis*, and *Aspergillus niger* from the blowholes of bottlenose dolphins (*Tursiops truncatus*)

Yoshito OHNO[1)], Yuichiro AKUNE[1)], Yasuo INOSHIMA[2)] and Rui KANO[3)]*

[1)]Port of Nagoya Public Aquarium, 1-3 Minato-machi, Minato-ku, Nagoya, Aichi 455-0033, Japan
[2)]Cooperative Department of Veterinary Medicine, Gifu University, 1-1 Yanagido, Gifu, Gifu 501-1193, Japan
[3)]Department of Veterinary Pathobiology, Nihon University College of Bioresource Sciences, 1866 Kameino, Fujisawa, Kanagawa 252-0880, Japan

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ABSTRACT. Pulmonary mycosis is a fungal disease that commonly affects bottlenose dolphins (*Tursiops truncatus*) and is generally treated by the oral administration of azoles, such as itraconazole (ITZ) and voriconazole (VRZ). However, antifungal susceptibility testing of clinical isolates has not been well performed as a routine clinical examination in aquaria. In this study, we collected fungal species from the blowholes of 14 bottlenose dolphins, of which 12 were treated with ITZ or VRZ. All dolphins were housed in the Port of Nagoya Public Aquarium. The fungal species *Candida albicans*, *C. tropicalis*, *C. glabrata*, *Aspergillus fumigatus*, and *A. niger* were isolated. E-tests were performed to determine the minimum inhibitory concentrations (MICs) of ITZ and VRZ on these isolates. VRZ-resistant *C. tropicalis* (MIC: >32 μg/ml) and *A. niger* (MIC: >32 μg/ml) were isolated from three dolphins treated with ITZ or VRZ. Additionally, azole-resistant isolates of *C. albicans* and *C. glabrata* were collected from two dolphins that had never received azole therapy. To the best of our knowledge, our study is the first to report the isolation of VRZ-resistant *C. albicans*, *C. tropicalis*, and *A. niger* from the blowholes of bottlenose dolphins. Thus, antifungal susceptibility testing is a crucial strategy for selecting antifungal agents to treat respiratory fungal infections in bottlenose dolphins in aquaria.

KEY WORDS: *Aspergillus*, bottlenose dolphin, *Candida*, susceptibility testing, voriconazole-resistant

Pulmonary mycosis is a fungal infection that commonly affects bottlenose dolphins (*Tursiops truncatus*) and is most often caused by *Candida* spp. or *Aspergillus* spp. [4, 18]. Various antifungal compounds, such as flucytosine, fluconazole (FLZ), itraconazole (ITZ), voriconazole (VRZ), posaconazole (PSZ), amphotericin B (AMB), and micafungin (MCF), are used to treat respiratory fungal infections in bottlenose dolphins [15, 17, 18]. AMB exhibits broad-spectrum antifungal activity, however, intravenous administration of liposomal AMB reportedly induced renal dysfunction in a bottlenose dolphin [16]. MCF, a semi-synthetic echinocandin, is an effective antifungal against *Candida* spp. and *Aspergillus* spp., however, intravenous administration of MCF in a dolphin reportedly caused leukopenia as a side effect [15]. Therefore, pulmonary mycosis in bottlenose dolphins is generally treated by the oral administration of azoles, such as ITZ and VRZ, which are effective against fungal infections and cause few side effects [17].

Azole-resistant fungi were isolated from human patients receiving azole therapy [1, 7, 14, 21] and could be isolated from bottlenose dolphins undergoing long-term antifungal therapy. Therefore, antifungal susceptibility testing of clinical isolates in aquaria could be useful for the identification of suitable antifungal agents for dolphins with respiratory fungal infections.

We isolated fungal species from the blowholes of 14 bottlenose dolphins housed in the Port of Nagoya Public Aquarium (PNPA) and determined the minimum inhibitory concentrations (MICs) of ITZ and VRZ on these isolates by conducting E-tests.

MATERIALS AND METHODS

Animals

A total of 14 bottlenose dolphins housed in the PNPA were examined in this study. Information on the dolphins and antifungal

*Correspondence to: Kano, R.: kanou.rui@nihon-u.ac.jp

medications is shown in Table 1. Because 12 of the 14 dolphins showed clinical signs (malodorous breath, fever, or sputum discharge) and abnormalities (neutrophilia, elevated fibrinogen, elevated erythrocyte sedimentation rate, or leukocytosis), they were treated with ITZ (Itraconazole, Nichi-Iko Pharmaceutical, Toyama, Japan; 2.0–2.5 mg/kg, *bis in die* [BID], *per os* [PO]) or VRZ (VFEND®, Pfizer Japan, Tokyo, Japan). During the VRZ medication period, we adopted an intermittent medication regimen (loading dose 1.0–2.4 mg/kg, BID, PO, for the first 3 consecutive days; maintenance dose 1.0–3.1 mg/kg, BID, PO, every 7–10 days) and monitored plasma concentrations in order to avoid toxic VRZ levels, based on the different pharmacokinetics of VRZ and its longer half-life in this species than in humans [5].

Mycological examination

Each blowhole sample was collected directly onto a Sabouraud dextrose agar plate (BD Japan, Tokyo, Japan) through the training of a forced exhalation, as described previously [6]. After sampling, the plate was incubated at 37°C for 48 hr in an IC-450A incubator (AS ONE Corp., Osaka, Japan). Fungal colonies were then identified as *Candida* spp. or *Aspergillus* spp. based on the sequence homology of the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2), as described previously [9]. 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].

Antifungal susceptibility testing

E-tests were performed to determine the MICs of ITZ and VRZ for the 20 isolates of *Candida* spp. or *Aspergillus* spp. collected from the blowholes of 14 dolphins, as described in the E-test Technical Guide 10 (bioMérieux Japan, Tokyo, Japan).

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, prepared by the Clinical Laboratory Standards Institute (CLSI) [3]. The MICs of ITZ on S, S-DD, and R isolates were determined to be ≤0.125 μg/m*l*, 0.25–0.5 μg/m*l*, and ≥1 μg/m*l*, respectively. The MICs of VRZ on S and R isolates were determined to be ≤1 μg/m*l* and ≥4 μg/m*l*, respectively [3]. *Aspergillus* spp. were classified as S, S-DD, or R to ITZ and VRZ according to the clinical breakpoints in the M38-A2 CLSI guidelines [3]. The MICs of ITZ and VRZ on S, S-DD, and R isolates were determined to be ≤1 μg/m*l*, 2 μg/m*l*, and ≥4 μg/m*l*, respectively [3].

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

Dolphin number	Sex	Age (years)[a)]	Clinical sign[b)]	Abnormality[c)]	Medication[d)]	Date[e)]	Strain number
1	Female	17	–	–	–	2013/08/07	1
		17	–	–	–	2013/08/07	2
		20	–	–	–	2016/06/06	3
2	Female	16	M	N	ITZ[f)] (83; 16)	2013/08/07	4
		19	–	–	–	2016/06/14	5
3	Female	19	M, F	N, eFIB, eESR	ITZ (24; −2)	2016/05/27	6
4	Female	3	M, F	N, eFIB, eESR	VRZ[g)] (54; −109)	2017/08/04	7
5	Female	16–17	M, F, S	N, eFIB, eESR, L	VRZ (139; 139)	2017/08/16	8
6	Female	22	M, F, S	N, eFIB, eESR, L	VRZ (112; +9)	2017/08/10	9
7	Female	11–14	–	–	–	2013/08/07	10
		11–14	–	–	–	2013/08/07	11
		11–14	–	–	–	2013/08/07	12
		14–17	–	–	–	2016/05/27	13
8	Female	19	M, S	N, eFIB, eESR	VRZ (256; 199)	2015/05/07	14
9	Female	15–16	M, F, S	N, eFIB, eESR, L	VRZ (134; 32)	2016/12/13	15
10	Male	2 months	M, F, S	N, eFIB, eESR, L	VRZ (86; −4)	2016/10/24	16
11	Male	17	M, F, S	N, eFIB, eESR, L	VRZ (46; +38)	2014/06/12	17
12	Female	14–17	M, F, S	N, eFIB, eESR, L	VRZ (220; 140)	2015/05/10	18
13	Female	14–17	M, F, S	N, eFIB, eESR, L	VRZ (66; −11)	2015/05/09	19
14	Male	21	M, S	N, eFIB, eESR, L	ITZ (31; +42)	2016/06/12	20

a) Estimated age, except dolphin nos. 4 and 10, at the time of blowhole sampling. b) Clinical signs before medication; M: malodorous breath; F: fever; S: sputum discharge. c) Hematological abnormalities before medication; N: neutrophilia; eFIB: elevated fibrinogen; eESR: elevated erythrocyte sedimentation rate; L: leukocytosis. d) Figures in parentheses show the duration of medication and the day of blowhole sampling before (−), during, or after (+) medication. e) Figures show the date (year/month/day) of blowhole sampling. f) ITZ: itraconazole. g) VRZ: voriconazole.

RESULTS

Mycological examination

We isolated five fungal species (20 isolates) from the blowholes of 14 dolphins: three species of yeast (*Candida albicans*, *C. tropicalis*, and *C. glabrata* [6, 3, and 4 isolates, respectively]), and two species of filamentous fungi (*Aspergillus fumigatus* and *A. niger* [6 and 1 isolates, respectively]) (Table 2).

Antifungal susceptibility testing

One isolate of *C. albicans* (strain no. 6) and two isolates of *C. tropicalis* (nos. 8 and 9) were R to both azoles (ITZ and VRZ: >32 μg/m*l*). Three isolates of *C. glabrata* (nos. 10, 11, and 12) were R to ITZ (MIC range: 1.5–2.0 μg/m*l*). Three isolates of *C. albicans* (no. 5), *C. tropicalis* (no. 7), and *C. glabrata* (no. 13) were S-DD to ITZ (MIC range: 0.19–0.25 μg/m*l*), respectively (Table 2). Four of the ten isolates (nos. 6, 10, 11, and 12) were R to each azole, despite pre- or non-azole therapy. One isolate of *C. albicans* (no. 4) was S to both azoles, despite post-azole therapy (Tables 1, 2).

All six *A. fumigatus* isolates were S to both azoles (MIC range of ITZ: 0.047–0.75 μg/m*l*; MIC range of VRZ: 0.025–0.64 μg/m*l*); however, *A. niger* (no. 20) was only R to VRZ (MIC: >32 μg/m*l*) (Table 2).

Table 2. Antifungal susceptibilities of twenty isolates to itraconazole (ITZ) and voriconazole (VRZ) determined by E-test

Strain number	Species	Minimum inhibitory concentrations (μg/m*l*)	
		ITZ	VRZ
1	*Candida albicans*	0.032	0.003
2	*Candida albicans*	0.047	0.008
3	*Candida albicans*	0.047	0.012
4	*Candida albicans*	0.023	0.008
5	*Candida albicans*	0.190	0.125
6	*Candida albicans*	>32	>32
7	*Candida tropicalis*	0.250	0.125
8	*Candida tropicalis*	>32	>32
9	*Candida tropicalis*	>32	>32
10	*Candida glabrata*	2	0.047
11	*Candida glabrata*	1.5	0.064
12	*Candida glabrata*	1.5	0.064
13	*Candida glabrata*	0.190	0.023
14	*Aspergillus fumigatus*	0.250	0.094
15	*Aspergillus fumigatus*	0.047	0.064
16	*Aspergillus fumigatus*	0.750	0.025
17	*Aspergillus fumigatus*	0.750	0.640
18	*Aspergillus fumigatus*	0.250	0.064
19	*Aspergillus fumigatus*	0.250	0.047
20	*Aspergillus niger*	1	>32

DISCUSSION

Previous studies have reported FLZ- or ITZ-resistant *C. albicans* and *C. tropicalis*, and azole-resistant (ITZ, VRZ, and PSZ) *A. fumigatus* isolates from the respiratory tracts of bottlenose dolphins [2, 22]. To our knowledge, this is the first report of VRZ-resistant *C. albicans*, *C. tropicalis*, and *A. niger* isolates from the blowholes of bottlenose dolphins. Based on our findings, we suspect that the frequency of azole-resistant pathogenic fungi is increasing in dolphins and their aquarium environments.

VRZ has recently been used to treat respiratory fungal infections in bottlenose dolphins [15, 17]. In human patients, the acquisition of azole-resistance in *Candida* spp. and *Aspergillus* spp. can occur with widespread [21] or long-term antifungal therapy [1, 7, 14]. Therefore, a similar process is likely to occur in bottlenose dolphins housed in aquaria. VRZ-resistant *C. tropicalis* (nos. 8 and 9) and *A. niger* (no. 20) were isolated from azole-treated dolphins (nos. 5, 6, and 14) (Tables 1, 2).

In this study, we used ITS region analysis to identify fungal species. However, recent reports show that cryptic *Aspergillus* spp. are found in clinical and environmental isolates [8, 23] and that it is difficult to distinguish the cryptic species correctly by only morphological or ITS region analysis [23]. Therefore, the *A. niger* isolate (no. 20) was sensu lato and might be one of the species in the *Aspergillus* section *Nigri* (e.g. *A. tubingensis*), as described previously [8, 23]. Additionally, it is reported that the cryptic species, especially *A. tubingensis*, have low susceptibility (resistance) to azoles (ITZ and VRZ) [8, 10]. Consequently, it is crucial to identify the specific species, including cryptic *Aspergillus* spp., by using combination analysis of another gene region (e.g. β-tubulin or calmodulin) for the accurate selection of antifungal agents in bottlenose dolphins.

Fungal azole resistance could be caused by altered sterol biosynthesis (e.g. mutations in sterol 14α-demethylase lowering its affinity for the drug), target gene upregulation (e.g. *ERG11*/*CYP51A* encoding sterol 14α-demethylase), or increased efflux (e.g. overexpression of genes encoding the membrane proteins of the ABC transporter [*CDR1/CDR2*] or major facilitator [*MDR1*] superfamilies) [11, 12, 24]. Therefore, the reduced susceptibility of clinical isolates to azoles in this study may result from *ERG11*/*CYP51A* mutations, or the upregulated expression of *ERG11*/*CYP51A* or efflux pumps.

Notably, strains of *C. albicans* (no. 6) and *C. glabrata* (nos. 10, 11, and 12) that were resistant to ITZ and/or VRZ, and strains of *C. tropicalis* (no. 7) and *C. glabrata* (no. 13) that had low susceptibility to ITZ were isolated from two dolphins (nos. 3 and 4) before they were treated with azoles and from an untreated dolphin (no. 7) (Tables 1, 2). A recent epidemiological study of *Candida* spp. showed that some non-albicans *Candida* spp. (e.g. *C. glabrata*) have innate resistance to azoles (specifically FLZ) [19] and also have lower susceptibility to azoles (FLZ and VRZ) than *C. albicans* [19]. Therefore, *C. glabrata* (nos. 10, 11, and 12) might have innate resistance to ITZ, and *C. tropicalis* (no. 7) and *C. glabrata* (no. 13) might have low susceptibility to ITZ. Moreover, Takahashi *et al*. [22] reported that azole-resistant *Candida* spp. are likely to spread to other dolphins from those infected with azole-resistant fungi or from contaminated pool water. Sidrim *et al*. [20] also reported that humans, domesticated animals, wild animals, and aquatic or terrestrial environments are likely to carry resistant microorganisms, which could spread to other animals and natural environments. Therefore, the azole-resistant *Candida* spp. (nos. 6, 10, 11, and 12) could spread between dolphins living in the same environment.

In conclusion, our study is the first to report VRZ-resistant *C. albicans*, *C. tropicalis*, and *A. niger* isolates from the blowholes

of bottlenose dolphins, regardless of whether they were treated with azoles. Moreover, antifungal susceptibility testing could be crucial for selecting antifungal agents to treat respiratory fungal infections in bottlenose dolphins in aquaria.

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REFERENCES

1. Bellete, B., Raberin, H., Morel, J., Flori, P., Hafid, J. and Manhsung, R. T. 2010. Acquired resistance to voriconazole and itraconazole in a patient with pulmonary aspergilloma. *Med. Mycol.* **48**: 197–200. [Medline] [CrossRef]
2. Bunskoek, P. E., Seyedmousavi, S., Gans, S. J. M., van Vierzen, P. B. J., Melchers, W. J. G., van Elk, C. E., Mouton, J. W. and Verweij, P. E. 2017. Successful treatment of azole-resistant invasive aspergillosis in a bottlenose dolphin with high-dose posaconazole. *Med. Mycol. Case Rep.* **16**: 16–19. [Medline] [CrossRef]
3. Cantón, E., Espinel-Ingroff, A. and Pemán, J. 2009. Trends in antifungal susceptibility testing using CLSI reference and commercial methods. *Expert Rev. Anti Infect. Ther.* **7**: 107–119. [Medline] [CrossRef]
4. Delaney, M. A., Terio, K. A., Colegrove, K. M., Briggs, M. B. and Kinsel, M. J. 2013. Occlusive fungal tracheitis in 4 captive bottlenose dolphins (*Tursiops truncatus*). *Vet. Pathol.* **50**: 172–176. [Medline] [CrossRef]
5. Ferrier, K. R. M., van Elk, C. E., Bunskoek, P. E. and van den Broek, M. P. H. 2017. Dosing and therapeutic drug monitoring of voriconazole in bottlenose dolphins (*Tursiops truncatus*). *Med. Mycol.* **55**: 155–163. [Medline] [CrossRef]
6. Frère, C. H., Krzyszczyk, E., Patterson, E. M., Hunter, S., Ginsburg, A. and Mann, J. 2010. Thar she blows! A novel method for DNA collection from cetacean blow. *PLoS One* **5**: e12299. [Medline] [CrossRef]
7. Fujita, S. 2007. Measurement of the minimal inhibitory concentrations and minimal fungicidal concentrations of antifungal agents against *Candida* strains isolated from blood cultures. *Jpn. J. Chemother.* **55**: 257–267.
8. Hashimoto, A., Hagiwara, D., Watanabe, A., Yahiro, M., Yikelamu, A., Yaguchi, T. and Kamei, K. 2017. Drug sensitivity and resistance mechanism in *Aspergillus* section *Nigri* strains from Japan. *Antimicrob. Agents. Chemother.* **61**: e02583–16. [Medline] [CrossRef]
9. Hinrikson, H. P., Hurst, S. F., Lott, T. J., Warnock, D. W. and Morrison, C. J. 2005. Assessment of ribosomal large-subunit D1-D2, internal transcribed spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important *Aspergillus* species. *J. Clin. Microbiol.* **43**: 2092–2103. [Medline] [CrossRef]
10. Howard, S. J., Harrison, E., Bowyer, P., Varga, J. and Denning, D. W. 2011. Cryptic species and azole resistance in the *Aspergillus niger* complex. *Antimicrob. Agents. Chemother.* **55**: 4802–4809. [Medline] [CrossRef]
11. Izumikawa, K., Tashiro, M. and Kohno, S. 2013. Current status of drug-resistant *Aspergillus*: evolution of resistance and future. *Jpn. J. Chemother.* **61**: 149–156.
12. Kakeya, H., Miyazaki, T., Miyazaki, Y. and Kohno, S. 2003. [Azole resistance in *Candida* spp]. *Nihon Ishinkin Gakkai Zasshi* **44**: 87–92 (in Japanese). [Medline] [CrossRef]
13. Kano, R., Sakai, M., Hiyama, M. and Tani, K. 2019. Isolation of *Aspergillus caninus* (synonym: *Phialosimplex caninus*) from a canine iliac lymph node. *Mycopathologia* **184**: 335–339. [Medline] [CrossRef]
14. Moudgal, V., Little, T., Boikov, D. and Vazquez, J. A. 2005. Multiechinocandin- and multiazole-resistant *Candida parapsilosis* isolates serially obtained during therapy for prosthetic valve endocarditis. *Antimicrob. Agents. Chemother.* **49**: 767–769. [Medline] [CrossRef]
15. Ohno, Y., Akune, Y., Nitto, H. and Inoshima, Y. 2019. Leukopenia induced by micafungin in a bottlenose dolphin (*Tursiops truncatus*): a case report. *J. Vet. Med. Sci.* **81**: 449–453. [Medline] [CrossRef]
16. Petermann, E. R., Townsend, F. I. Jr., Barger, P. C. and Newton, J. C. 2012. A retrospective look at a case of mucormycosis in a bottlenose dolphin (*Tursiops truncatus*) treated with liposomal amphotericin B and monitored with serum ELISA levels. pp. 42–43. *In*: 43rd Annual International Association for Aquatic Animal Medicine Conference Proceedings, Atlanta.
17. Reidarson, T. H., García-Párraga, D. and Wiederhold, N. P. 2018. Marine mammal mycoses. pp. 389–423. *In*: CRC Handbook of Marine Mammal Medicine, 3rd ed. (Gulland, F. M. D., Dierauf, L. A. and Whitman, K. L. eds.), CRC Press, Boca Raton.
18. Reidarson, T. H., McBain, J. F., Dalton, L. M. and Rinaldi, M. G. 2001. Mycotic diseases. pp. 337–355. *In*: CRC Handbook of Marine Mammal Medicine, 2nd ed. (Dierauf, L. A. and Gulland, F. M. D. eds.), CRC Press, Boca Raton.
19. Sandhu, R., Dahiya, S., Sayal, P. and Budhani, D. 2017. Increased role of nonalbicans *Candida*, potential risk factors, and attributable mortality in hospitalized patients. *J. Health. Res. Rev.* **4**: 78–83.
20. Sidrim, J. J. C., Carvalho, V. L., de Souza Collares Maia Castelo-Branco, D., Brilhante, R. S. N., de Melo Guedes, G. M., Barbosa, G. R., Lazzarini, S. M., Oliveira, D. C. R., de Meirelles, A. C. O., Attademo, F. L. N., da Bôaviagem Freire, A. C., de Aquino Pereira-Neto, W., de Aguiar Cordeiro, R., Moreira, J. L. B. and Rocha, M. F. G. 2016. Antifungal resistance and virulence among *Candida* spp. from captive *Amazonian manatees* and west Indian manatees: potential impacts on animal and environmental health. *EcoHealth* **13**: 328–338. [Medline] [CrossRef]
21. Sütçü, M., Acar, M., Genç, G. E., Kökçü, İ., Aktürk, H., Atay, G., Törun, S. H., Salman, N., Erturan, Z. and Somer, A. 2017. Evaluation of *Candida species* and antifungal susceptibilities among children with invasive candidiasis. *Turk. Pediatri. Ars.* **52**: 145–153. [Medline] [CrossRef]
22. Takahashi, H., Ueda, K., Itano, E. N., Yanagisawa, M., Murata, Y., Murata, M., Yaguchi, T., Murakami, M., Kamei, K., Inomata, T., Miyahara, H., Sano, A. and Uchida, S. 2010. *Candida albicans* and *C. tropicalis* isolates from the expired breathes of captive dolphins and their environments in an aquarium. *Vet. Med. Int.* **2010**: 349364. [Medline] [CrossRef]
23. Toyotome, T. 2016. Causative agents of aspergillosis including cryptic *Aspergillus* species and *A. fumigatus*. *Med. Mycol. J.* **57**: J149–J154. [Medline] [CrossRef]
24. Yamaguchi, H. 2004. The current status and challenges for antifungal susceptibility testing. *J. Jpn. Soc. Clin. Microbiol.* **14**: 129–145.