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Short Note

A Suggested In-House Respiratory Fungal Culture Protocol for Bottlenose Dolphins (*Tursiops truncatus*)

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The landscape of fungal infections in cetaceans has markedly changed over the last decades, representing a potential disruptor to conservation efforts (Reidarson et al., 2018). Several fungal species have already been reported in cetaceans, with some causing a severe impact on morbidity and mortality (Reidarson et al., 2018; Garcia-Bustos et al., 2024). The different fungal forms hold specific diagnostic and clinical significance as their distinct pathogenic mechanisms and host interactions carry different prognoses (Reidarson et al., 2018). These include molds (*Aspergillus* spp., fungi from the Order Mucorales, *Fusarium* spp., *Scedosporium prolificans*, *Cladosporium* spp., *Conidiobolus coronatus*, *Neoscytalidium dimidiatum*, *Trichophyton* spp.), yeasts and yeast-like organisms (*Candida* spp., *Cryptococcus* spp., *Malassezia* spp., *Trichosporon* spp.), and dimorphic fungi (*Histoplasma capsulatum*, *Blastomyces* spp., *Paracoccidioides* spp., *Sporothrix schenckii*, *Coccidioides* spp.) (Migaki et al., 1978; Cates et al., 1986; Gugnani, 1992; Jensen et al., 1998; Miller et al., 2002; Zalar et al., 2007; Staggs et al., 2010; Elad et al., 2011; Bunskoek et al., 2017; Simeone et al., 2017; Ueda et al., 2017; Reidarson et al., 2018; Wan et al., 2018; Maldonado et al., 2019; Marques et al., 2021; Teman et al., 2021; Kanegae et al., 2022; Pacheco-Polanco et al., 2023; Garcia-Bustos et al., 2024).

Fungal infections in both human and veterinary medicine are frequently underemphasized despite causing considerable morbidity and mortality (Reidarson et al., 2018; Wiederhold, 2021). A study by Denning (2024) estimates that 6.5 million human cases of invasive fungal infections and 3.8 million fatalities occur annually worldwide. Considering their role in public health, the World Health Organization (WHO) (2022) recently released the first global effort to systematically prioritize fungal pathogens, targeting the improvement of the overall response to the emergence of fungal pathogens.

The development of resistances to currently available antifungals has outpaced the availability of new drugs, which represents a crucial topic from a One Health perspective, illustrated, for example, by the use of fungicides in agriculture and flower production, which dampen clinical drug efficacy (Pereira et al., 2021; Wiederhold, 2021; Fisher et al., 2022; Woods et al., 2023). Other factors contributing to the increase of antifungal resistance include the erroneous clinical management of fungal infections (e.g., empirical and/or repeated or long-term therapies, poor compliance), the change of biotic interactions, the mechanisms of fungal intrinsic virulence, and the overall change in climate, which may alter the geographical range of fungi and adaptive resistances (Perlin et al., 2017; Fisher et al., 2022).

Pneumonia is one of the most common diseases among managed and free-ranging bottlenose dolphins, and pulmonary fungal infections are reported to be the most common mycoses in dolphins (Venn-Watson et al., 2012; Reidarson et al., 2018). Several factors can contribute to the development of respiratory fungal disease in cetaceans, including the anatomical and physiological specific features that enable pathogens to be easily introduced into the lower respiratory tract; the presence of underlying diseases or pollutants; previous pharmacological treatments (e.g., antibiotics and/or corticosteroids); stress, immune, and genetic factors; as well as major dirt upheavals that increase environmental fungal loads (Venn-Watson et al., 2012; Fahlman et al., 2015; Martony et al., 2019; Prakash & Chakrabarti, 2019).

The clinical presentation of fungal infections is commonly nonspecific, with minimal to no clinical signs in early stages, varying from an acute onset to a more chronic presentation (Reidarson et al., 2018; Mendonça et al., 2022). An early evaluation is essential to guarantee a timely diagnosis, prompt treatment, and the improvement of

the overall prognosis of certain fungal infections (Riwes & Wingard, 2012). This is crucial when dealing with poor prognosis infections, which have a rapid onset due to the high capacity of angioinvasion and tissue necrosis such as mucormycosis (Reidarson et al., 2018; Skiada et al., 2018). Different methods can be used for fungal infection diagnosis, from a conventional laboratory approach (e.g., cytology, culture, histopathology) to molecular-based methods. However, to achieve a more robust diagnosis, a combination of techniques should be used (Riwes & Wingard, 2012; Donnelly et al., 2019; Mendonça et al., 2022). Molecular methodologies allow efficient and accurate results in a significantly reduced turnaround time. Notwithstanding, fungal cultures are still the gold standard in diagnosing invasive pulmonary fungal infections, which are economical and readily available diagnostics compared to molecular methods (Kozel & Wickes, 2014; Donnelly et al., 2019; Mendonça et al., 2022).

Although managed cetaceans are under a preventive medicine program, specific fungal diagnostic testing is usually performed only after the onset of clinical signs or hematology or cytology abnormalities, which are low-sensitivity indicators of mycotic infections that may hamper a successful clinical approach. Considering the increasing prevalence of fungal disease, the emergence of certain fungal pathogens, and the high susceptibility of cetaceans to respiratory disease, it is crucial to develop a thorough fungal surveillance protocol to be included in their routine medical management (Staggs, 2017). For this, dolphinaria often work with external laboratories which, in the authors' experience, has proven to be particularly challenging. Due to the lack of mycology reference laboratories in Portugal and the low prevalence of fungal disease in small animals, leading standard laboratories to receive few to no fungal cases and, consequently, often reaching questionable or incomplete results (e.g., identification of *Candida* spp. only to the genus level, filamentous fungi commonly not reported and discarded as contaminants, no possibility of antifungal susceptibility testing), clinical management was often delayed. Accordingly, the authors developed an in-house fungal culturing protocol for a group of 26 bottlenose dolphins (*Tursiops truncatus*) housed at Zoomarine Portugal, with routine screening and follow-up. This protocol

Figure 1. Flowchart of the respiratory fungal culture protocol

seeks to offer standard in-house procedures easily included in the preventive medicine program that may be beneficial to other practitioners, allowing early and reliable results, ultimately permitting an effective medical management of fungal respiratory disease in cetaceans under professional care.

Mycology Protocol

The in-house respiratory culture protocol was implemented into the already established preventive medicine program and fungal surveillance approach, which included gathering of behavioral data; physical examination; monthly blood collection for complete blood count and biochemistry analyses; routine direct microscopy of sputum, gastric fluid, and fecal samples; and ultrasound examination.

The culturing protocol included a systematic and critical control approach in sampling, inoculation, incubation, fungal phenotypic identification, and susceptibility testing in which all steps were thoroughly scrutinized. All members of the veterinary team were aware of the protocol and actively participated in the collection of the samples, together with the bottlenose dolphins' keepers. With appropriate training in laboratory techniques, mycology procedures, and safety measures, the first author was responsible for the inoculation and incubation of samples as well as the fungal macroscopic and microscopic identification. A more comprehensive review of clinical mycology and laboratory techniques can be found in the works of Anaissie et al. (2009), Samanta (2015), and Walsh et al. (2018).

Sampling

In human medicine, sputum samples are frequently used to evaluate signs of respiratory disease or airway inflammation, including for microbiological investigation (Pashley et al., 2012). Contrarily to bronchoalveolar lavages (BALs), sputum sampling is non-invasive and more readily available (Pashley et al., 2012). However, human sputum samples are contaminated with oral microorganisms, which may require a careful division before culture, separating the plug from the saliva to minimize oropharyngeal contamination (Pashley et al., 2012; Samanta, 2015; Engel et al., 2020).

In cetaceans, while sputum samples are likewise unquestionably easier to obtain compared to BALs, the question of oropharyngeal contamination is overall dismissible given the anatomical separation between the respiratory and digestive systems. Furthermore, bottlenose dolphins' sputum may be of increased diagnostic value compared to humans since they exchange around 80% of their lung volume in one breath, associated with a rapid expulsion of cell-rich material from the deep respiratory tract (Sweeney et al., 2003; Varela et al., 2007).

The frequency of screening sputum sampling was decided every 3 mo, considering the practicality of the protocol among the other preventive medicine tests. Additional sampling was justified according to respiratory signs, hematological abnormalities, and/or presence of filamentous fungal elements in the weekly direct examination of sputum samples. Follow-up sampling throughout any antifungal treatments was done every 15 or 30 d, according to the fungal etiology and overall clinical data, as poor prognosis infections were given a more thorough follow-up.

For sampling, the first exhalation was discarded to minimize water contamination. The area around the blowhole was disinfected through the concentric circle technique, in a 15 cm radius, in the following order: chlorhexidine 1%, sterile water, and a dry sterile gauze. The bottlenose dolphin would then voluntarily exhale five times into a sterile container (e.g., Avantor VWR, Radnor, PA, USA), which was identified with the name of the animal. During the procedure, the other conspecifics would be in the far opposite end of the pool to minimize cross-contamination. The keeper collecting the sample was wearing gloves throughout the procedure after a thorough hand sanitation.

Screening sampling was postponed whenever environmental factors could increase exposure to airborne particles which represented an increasing source of contamination—for example, during rainy days or major dirt upheavals.

After sampling, blowhole specimens were refrigerated at 8ºC until inoculation. There is a lack of consensus concerning the effect of refrigeration on fungal specimens, although samples potentially contaminated with bacteria should be stored refrigerated at 4º to 8ºC, especially when processing is to be delayed (Bhargava, 2019).

Inoculation and Incubation

Inoculation of each sputum sample was performed up until 4 h after sampling as fungal viability may decrease with prolonged storage (Bhargava, 2019). To increase the number of filamentous colonies and overall culture sensitivity, the entirety of the sputum sample was collected from the container using a sterile pipette and deposited in the culture plates (Pashley et al., 2012; Fraczek et al., 2013).

Each sample was inoculated through the quadrant streaking technique, first on Sabouraud Dextrose Chloramphenicol Agar (SDCA; Frilabo, Maia, Portugal) and then on CHROMagar™ Candida Plus (CC-Plus; Frilabo). All plates were identified with the date and name of the dolphin, and parafilm was wrapped one time around the circumference of each plate to minimize contamination.

SDCA has a high concentration of dextrose (40 g/L), which in addition to the low pH (5.4 to 5.8) promotes the growth of both yeasts and molds, as well as the formation of spores (conidia and sporangiospores). The incorporation of chloramphenicol into the Sabouraud agar inhibits bacterial growth (Merck Millipore, n.d.).

CC-Plus is a selective chromogenic culture media that allows a qualitative direct detection and differentiation of five different *Candida* species: (1) *Candida albicans* (green-blue), (2) *Candida glabrata* (mauve), (3) *Candida tropicalis* (metallic blue), (4) *Candida krusei* (pink and fuzzy), and (5) *Candida auris* (light blue with a blue halo). The absence of growth in CC-Plus does not preclude the presence of other species of *Candida* (CHROMagar™, n.d.).

The SDCA plates were incubated at 25ºC and observed daily for the development of fungal colonies. In the case of yeast growth, the incubation period could be as short as 24 to 72 h. Although faster-growing molds (e.g., mucormycetes, *Aspergillus* spp.; Figure 2) can grow in 24 to 48 h, slow-growing species may require a longer incubation period (Engel et al., 2020). A study by Morris et al. (1996) describing fungal development in 2,173 cultures reported a 94% fungal detection by day 7 and a 98% detection by day 14 of incubation. Accordingly, the incubation of filamentous fungi lasted at least 72 h, but they were commonly left for 15 d. CC-Plus plates were incubated at 37ºC for 36 to 48 h due to the mentioned yeast rapid growth.

Identification

The phenotypic identification of filamentous fungi was achieved through macroscopic colonial appearance (growth rate/diameter, topography, texture, surface and reverse color, diffusible pigment presence, and color) and micromorphology fungal features. Microscopic identification included the observation of hyphae (color, size, septation, and structure) and conidia (septation, shape, size, color, wall texture, arrangement, type, and growth of conidiogenous cells).

For microscopic identification of filamentous fungi, a piece of scotch tape was gently pressed onto the surface of the fungal colony in the opened culture plate and then placed on a previously prepared microscope slide with three drops of Lactophenol cotton blue (LPCB; Avantor VWR). According to the dimensions of the fungal colony, sampling was done on the mid-area between the center and the periphery of the colony. Two more drops of LPCB were added above the scotch tape, which was then covered with a 24 × 50 mm coverslip. This last step was optional for microscopical observation; however, the coverslip may help disperse any occasional air bubbles on the scotch tape and facilitates the examination of the fungal micromorphology under the light microscope (100×, 400×).

Yeast colonies on SDCA were also observed for color, texture, shine, consistency, and border characteristics. Yeasts were prepared for microscopic observation using a sterile, disposable inoculation loop to transfer a portion of the colony to a

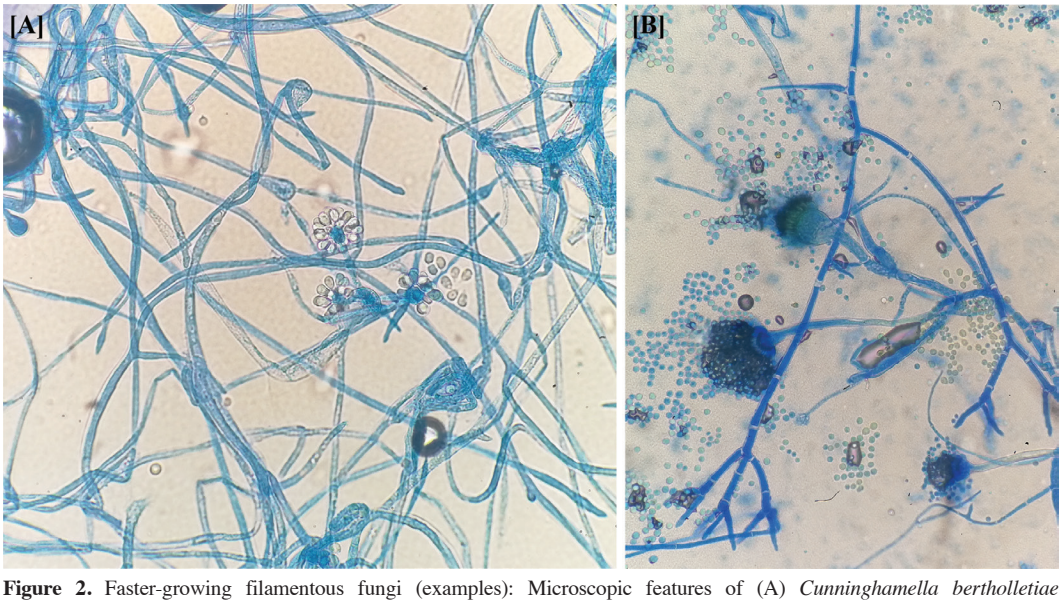

Figure 2. Faster-growing filamentous fungi (examples): Microscopic features of (A) *Cunninghamella bertholletiae* (mucormycete) and (B) *Aspergillus fumigatus* (LPCB, ×400) after 48 h of incubation at 25ºC in SDCA.

previously prepared slide with one drop of LPCB, which was then added a 22 × 22 mm coverslip. Yeast observation was also done under 100× and 400× magnification to examine their shape and size. As previously mentioned, the color and texture of the colonies on CC-Plus permitted the identification of *Candida* spp.

A mycology report card was developed according to the macroscopic and microscopic characteristics to evaluate for the identification of the species, which was filled accordingly for each culture (Table 1). A fungal atlas was consulted whenever needed (Walsh et al., 2018).

Antifungal Susceptibility Testing

Antifungal susceptibility testing (AFST) was further initiated to better guide occasional antifungal treatments as well as for epidemiological surveillance of emerging resistances. Broth microdilution reference techniques, developed by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) and by the Clinical and Laboratory Standards Institute (CLSI) (Rex et al., 2008a, 2008b; Guinea et al., 2022, 2023), are defined for AFST in yeasts and molds. However, these reference techniques are time-consuming and more adapted for reference laboratories. Commercially available methods, such as the Etest strip technique,

Table 1. Mycology report card for fungal phenotypical identification

SAMPLE IDENTIFICATION	
Animal	Name	_____ Species	Bottlenose dolphin ☐ Other:
Biological sample	Sputum ☐ Other:
Date	_____
Ongoing treatment	_____
Comments	_____
FUNGAL IDENTIFICATION	
Direct examination	Observation dates _____ Without fungal findings ☐ Fungal load (+, ++, +++) Yeasts ☐ Hyphae ☐ Pseudohyphae ☐ Mycelia ☐ Spores ☐ Hyaline ☐ Pigmented ☐
Macromorphology	Observation dates _____ Culture medium _____ **Yeast/yeast-like //** Fungal load (0, +, ++, +++) Colony diameter _____ Pigmentation _____ Borders: regular ☐ irregular ☐ Texture: smooth ☐ rough ☐ Shine: shining ☐ opaque ☐ Consistency: creamy ☐ mucous ☐ Other: **Filamentous //** Fungal load (0, +, ++, +++) Colony diameter _____ Surface pigmentation _____ Reverse_____ Borders (regular, irregular, radiated) Texture (granular, flaky, powdery, velvety) Topography (flat, convex, pleated, cerebriform, umbilicate) Other:
Micromorphology	Observation dates _____ **Yeast/yeast-like** // Shape (circular, oval, elongated), Size, Hyphae/Pseudohyphae?_____ **Filamentous //** Hyphae: coenocytic, septated, poorly septated? Narrow/wide? Ramification angle _____ Hyaline/pigmented? Macro/microconidia? Conidia in clusters or chains, septation, texture, and shape (among others)? Brush? *Aspergillus* spp. head (and specifications)? Sporangia, rhizoids (among others)?
Susceptibility testing	Exam dates _____ Results _____
Diagnosis	

are commonly used as a practical alternative as they are ready-to-use methods, which guarantee similar results to the reference techniques (Dannaoui & Espinel-Ingroff, 2019). The Etest strip technique comprises a predefined concentration gradient of a specific antifungal agent which is used to determine the minimum inhibitory concentration (MIC) of the tested organism in µg/mL (bioMérieux SA, 2013). Studies evaluating the correlation between the gradient strips and the broth microdilution techniques take into consideration clinical breakpoints or, when not available, epidemiological cutoff values determined by the EUCAST or CLSI techniques (Dannaoui & Espinel-Ingroff, 2019).

Antifungal susceptibility testing of both yeasts and molds was performed using antifungal impregnated strips of itraconazole, fluconazole, and voriconazole (Liofilchem, Abruzzi TE, Italy), as these were the most common antifungal agents prescribed in the authors' experience. Yeast colonies from a 48-h pure culture on SDCA were homogenized in sterile water to obtain a turbidity equivalent to 0.5 McFarland standard. A mature growth of 5 to 7 d was necessary for filamentous fungal suspensions. For inoculation, a sterile swab was soaked in the inoculum suspension, removing excess fluid by pressing the swab against the inside wall of the test tube. The sample was evenly inoculated three times, in three directions, on the entire surface agar of RPMI 1640 MOPS plates (Liofilchem). The swab was soaked again before repeating the streaking procedure a second time. The antifungal strip was applied with the help of sterile forceps onto the agar surface, not touching the antifungal impregnated side of the strip, and only after the surface of the agar was completely dry. Incubation time at 35ºC was variable, depending on the species tested. For yeasts and some molds (e.g., *Aspergillus* spp., mucormycetes), a 48-h period was generally adequate, while an extended incubation period was needed for others. Plates were inspected daily for growth and presence of an inhibition ellipse. When testing azoles against yeasts, a trailing phenomenon can be observed as a lawn of microcolonies in the inhibition ellipse (bioMérieux SA, 2013). For yeasts, the MIC endpoint is at 80% inhibition, while for filamentous fungi, the reading endpoint is at 100% inhibition, the latter with a sharp and clear ellipse (bioMérieux SA, 2011).

Antifungal susceptibility testing was used before starting any antifungal treatment, as well as throughout therapy, as there may be a shift in the resistance pattern of the causative agent, especially considering the common need for long antifungal therapies. The choice of the antifungal drug considered AFST results, as well as medical history and clinical experience with previous infections by a certain fungal pathogen and its response to antifungal therapy.

General Considerations

The European Organization for Research and Treatment of Cancer and the Mycoses Study Group (EORTC/MSG) education criteria for invasive fungal disease classifies an infection into possible, probable, or proven, according to host factors, clinical features, and mycological evidence (Donnelly et al., 2019). The criteria for the diagnosis of proven invasive fungal infections differ according to the fungal etiology (molds, yeasts, *Pneumocystis* spp., or endemic mycoses). These criteria include histopathologic, cytopathologic, direct examination, and culture of specimens, as well as blood cultures, serological tests, and tissue nucleic acid analysis. Similarly, probable invasive fungal diseases are defined by host, clinical, and mycological criteria, specific to each fungal infection (e.g., aspergillosis, other pulmonary mold diseases, candidiasis, cryptococcosis, pneumocystosis, endemic mycoses). These consensus definitions, although developed for human medicine, may provide a basis for diagnosing cetacean fungal infection. In the authors' experience, assessment of treatment needs was adapted on an individual basis through a multidiagnostic approach in which culture results were correlated with the overall clinical situation (i.e., behavioral data, medical history, blood analysis, imaging), direct microscopy, and other indicators of invasive fungal disease, such as galactomannan and β-d-Glucan testing.

From a preventive medicine perspective, direct observation of blowhole specimens beforehand allowed the identification of fungal elements (i.e., conidia, hyphae, pseudohyphae, mycelia) and general signs of inflammation. Moreover, direct microscopy may provide a rapid fungal diagnosis and/or indicate the need for special culturing media. It may also determine the significance of an organism later identified in the culturing process. For example, if the cultured fungal organism is rarely pathogenic, but is observed in great numbers on direct examination, a disease process may be involved rather than being present merely as a contaminant (Walsh et al., 2018). Furthermore, as fungal spores may be ubiquitous in the environment, a single positive culture may not be accurate; thus, repeated cultures need to be considered.

A statistical analysis of the epidemiology of fungi in this collection of bottlenose dolphins falls beyond the scope of this work; however, in the authors' 3 y of experience, the in-house fungal culture protocol was easily included as part of the medical surveillance program, allowing a prompt diagnosis and thorough monitoring of proven respiratory infections by agents such as *Cunninghamella bertholletiae*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Candida albicans*, and *Candida glabrata*. Possible respiratory infections

by *Penicillium purpurogenem* and *Trichosporon mucoides* were also reported.

This point-of-care diagnostic testing may be beneficial for other dolphinaria who face the challenge of an early and reliable clinical diagnosis due to the lack of external laboratories that offer mycology services in their geographic area. Moreover, it is common for shipping periods to outside laboratories to be superior to 24 to 48 h in contrast with the possibility of immediate culturing using this in-house protocol. Also, while laboratories need to wait for the end of the described incubation period (15 d or more in the case of filamentous fungi) to emit an official report, in-house cultures permit a daily evaluation by the practitioner, which may anticipate further diagnostics of poor prognosis infections. This protocol presents a practical approach to evaluate the epidemiology of fungal infections in managed cetaceans but does not dismiss the need for appropriate training in laboratory and microbiology techniques.

Given the high prevalence of respiratory fungal disease in cetaceans, this suggested protocol only considered sputum cultures, although it can be promptly adapted to other biological samples (e.g., gastric fluid, feces) and applied to other species housed in zoological institutions. The emergence and reemergence of fungal pathogens are increasing concerns from a One Health perspective, and as the effects of climate change and fungal adaptative thermotolerance develop rapidly, it is essential that the present protocol constantly evolves and adapts while new potential fungal pathogens are established.

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