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The use of posaconazole delayed-release tablets in the successful treatment of suspected mucormycosis in a bottlenose dolphin (*Tursiops truncatus*) calf

Gonçalo N. Marques *, Nuno U. Silva, Miriam O. Leal, Carla A. Flanagan

Zoomarine Portugal, N125 KM 65, 8201-864, Guia, Portugal

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ABSTRACT

Mucorales infections in cetaceans have a high mortality rate. This case report refers to a bottlenose dolphin calf with suspected mucormycosis treated with posaconazole. This antifungal agent was discontinued after 96 days of therapy, however, the infection relapsed. Posaconazole was then resumed for a total of 255 days, with no signs of disease reactivation. The retrospective analysis of posaconazole serum levels in this successful case showed concentrations varying between 5.18 and 11.63 mg/L.

1. Introduction

Several fungi species are described to cause invasive infections in dolphins [1]. Mucormycosis is a rare infection caused by the ubiquitous fungi from the order Mucorales and is characterized by angioinvasion, thrombosis and tissue necrosis. Based on the anatomical site of involvement, mucormycosis in humans is classified into rhino-orbital-cerebral, pulmonary, cutaneous, gastrointestinal and disseminated forms [2]. As this infection may be rapidly fatal, it is crucial to have a timely diagnosis and perform an aggressive treatment [3].

Historically, most dolphins diagnosed with mucormycosis have not survived despite treatment [1]. We describe the successful management of a fungal infection in a bottlenose dolphin calf, in which *Cunninghamella bertholletiae* was identified. This clinical case explores the difficulties of the clinical management in calves and broadens the knowledge of a multimodal mucormycosis approach in cetaceans. This included the use of posaconazole in its delayed-release tablet formulation, one of the few antifungal agents with activity against Mucorales. After a 96-day posaconazole course and given the lack of evidence of an on-going infection, medication was discontinued. Fifty-five days after its suspension and given the evidence of the infection relapse, a second posaconazole course was initiated (day 150), with no signs of disease reactivation until the end of therapy (day 405). This case outlines the first documentation of a successful mycosis management in a marine mammal calf treated with delayed-release tablets of posaconazole.

2. Case

A male bottlenose dolphin calf, housed in an outdoor dolphinarium at Zoomarine Portugal, developed several leukocytosis episodes over its first months of life. Different antimicrobial treatments were given throughout this period and although the total white blood cell (WBC) count decreased after therapy, leukocytosis was recurrent. During this period, aetiology was never confirmed.

At one year of age, as part of a routine check-up, a direct microscopic faecal evaluation revealed the presence of coenocytic hyphae (Fig. 1). Simultaneously, a complete blood count showed a mild leukocytosis (10.0×10^9 WBC/L). Sputum sample evaluation was unremarkable.

After reported harsh breaths, thoracic radiographs were performed, showing a slight bronchoalveolar pattern of the left and right pulmonary apexes. Bronchoscopy images were compatible with a severe fungal infection, with multiple whitish prominent lesions, diffusely distributed on the tracheal and bronchial submucosa and mucosa.

From the faecal sample, *Cunninghamella bertholletiae* was identified through conventional PCR and sequencing (INSA - Instituto Nacional de Saúde Doutor Ricardo Jorge). Sensitivity testing was performed through the Epsilometer-test, with a posaconazole minimum inhibitory concentration (MIC) of 1.0 mg/L and an amphotericin B MIC of >32 mg/L.

Delayed-release tablets of posaconazole (5 mg/kg, PO, BID) were initiated (day 0) along with liposomal amphotericin B nebulisations (20–25 mg, BID) and silymarin supplementation (140 mg, PO, TID). After beginning posaconazole administration, nausea episodes and hyporexia were reported and medication compliance was revealed

* Corresponding author.
E-mail address: gnmarques1205@hotmail.com (G.N. Marques).

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difficult. A change of the posaconazole dosage regimen to 10 mg/kg, PO, SID (day 9) led to an immediate improvement, including an increase in appetite.

Overall, routine biochemistry analysis showed a slight increase of alanine aminotransferase (ALT) and aspartate transaminase (AST) after initiating posaconazole therapy but they remained within reference intervals throughout treatment. Serum iron and phosphatase alkaline (ALP) values began to show a gradual decreasing tendency (AQUALAB – Laboratório Clínico e de Saúde Pública).

In a follow-up bronchoscopy (day 47), lesions seen in the previous endoscopic exam were no longer observed, but a mildly hyperaemic tracheal mucosa was noted and nebulisations with amphotericin B were suspended. Given the negative results in both culture and direct microscopic evaluation of faecal and sputum samples, posaconazole was discontinued after 96 days of therapy.

A third follow-up bronchoscopy (day 132) showed that although the tracheal mucosa appeared healthy with no fungi plaques, whitish punctiform lesions were identified on the tracheal submucosa, decreasing distally and almost disappearing on the primary bronchi. Simultaneously, hyphae morphologically similar to the previous findings began to appear in both faecal and sputum direct microscopic evaluation.

Administration of posaconazole was resumed (day 150) at the same dosage (10 mg/kg, PO, SID) 55 days after it was discontinued.

Leukocytosis was a common finding throughout this second course of treatment as were low levels of serum iron, reaching values of 23.8 $\times$ 10^9 WBC/L and 32 μg/dL, respectively. The calf's appetite was inconsistent with occasional episodes of nausea and hyporexia.

Follow-up endoscopic exams showed only minor scar lesions on the tracheal and bronchial mucosa. From day 367, there was a clear and overall improvement of inflammatory markers, as well as in appetite and general behaviour. The second course of posaconazole was discontinued after 255 days of therapy.

Up until the time of writing, more than one year after posaconazole suspension, there have been no signs of an infection relapse. A thorough routine monitoring programme is maintained, which includes blood analysis, faecal and sputum direct microscopic evaluation and culture, and bronchoscopy.

Posaconazole serum concentrations throughout treatment were retrospectively measured using ultra-performance liquid chromatography-tandem mass spectrometry (Laboratory of the Clinical Pharmacy of the University Medical Center Utrecht, The Netherlands) – Fig. 2. Overall, considering both courses of posaconazole therapy, concentrations varied between 5.18 and 11.63 mg/L.

The first two measurements (day 3 and day 6) at a posaconazole regimen of 5 mg/kg, PO, BID showed concentrations of 6.26 and 5.18 mg/L, respectively. On day 13, four days after changing the regimen of posaconazole to 10 mg/kg, PO, SID, serum concentration was 5.48 mg/L and started to increase in the following measurements reaching levels of 10.17 mg/L in the first course of therapy.

After discontinuing the antifungal therapy, concentrations decreased rapidly, reaching values of 1.64 mg/L and <0.15 mg/L, 14 days and 29 days after stopping posaconazole therapy, respectively.

Two days after resuming posaconazole (day 152), there was a serum concentration of 6.38 mg/L and during this second period of therapy, later results were maintained between 8.5 and 11.63 mg/L in the serum samples analyzed.

3. Discussion

This report describes a long-term successful case of a fungal infection in a bottlenose dolphin calf, after a 96-day course of posaconazole and subsequent relapse of the mycotic infection. Given the subjective regimen of posaconazole therapy, the possibility of subtherapeutic drug concentrations as the cause for relapse was explored.

An important note concerns the fact that the biological sample in which the Mucorales species was identified (PCR) was a faecal sample, while lesions were observed in the respiratory system through bronchoscopy. The fungal specimen identified from the faecal sample may be of respiratory origin and thus being the organism responsible for the clinical infection. However, this was not directly confirmed and another fungal specimen may be eventually pointed as the aetiologic agent of the respiratory macroscopic lesions. The suboptimal sensitivity of both direct microscopy and culture of mucormycetes [3] should be considered as a plausible explanation for the unremarkable results on the evaluation of sputum samples in the initial approach of this clinical case.

Several aspects must be taken into consideration regarding antifungal therapy in a Mucorales infection. In this case, specifically, *Cunninghamella bertholletiae* was identified through molecular assays, which did raise extra concern, given the higher virulence associated with this fungal species [4]. Overall, the role of *in vitro* susceptibility testing for mucormycetes is not yet fully determined [5,6]. Not only are clinical breakpoints not available for Mucorales species, but epidemiological cut-off values (ECVs) are also not available for *C. bertholletiae*, which represented another hurdle in the choice of antifungal agents. Sensitivity testing results were then compared with the ECVs of *Lichtheimia corymbifera* (2 mg/L for both amphotericin B and posaconazole), another Mucorales species [6].

The role of amphotericin B, in this case, is uncertain, especially comparing the MIC results obtained with the available ECVs of *L. corymbifera*. Additionally, the simultaneous use of posaconazole is still questionable, with some reported contradictory interactions between

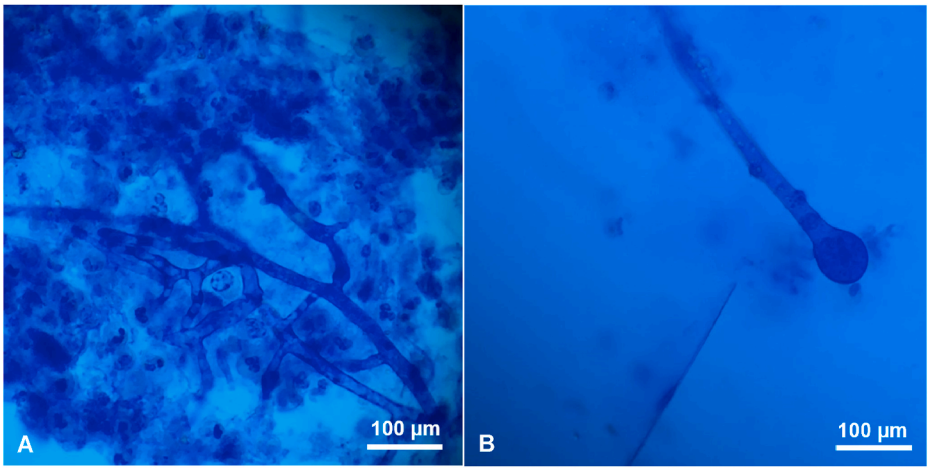

Fig. 1. Direct microscopy of a faecal sample (new methylene blue $\times$ 1000). A) coenocytic hyphae and leukocytes. B) Clearer view of the fungal specimen sporangiola.

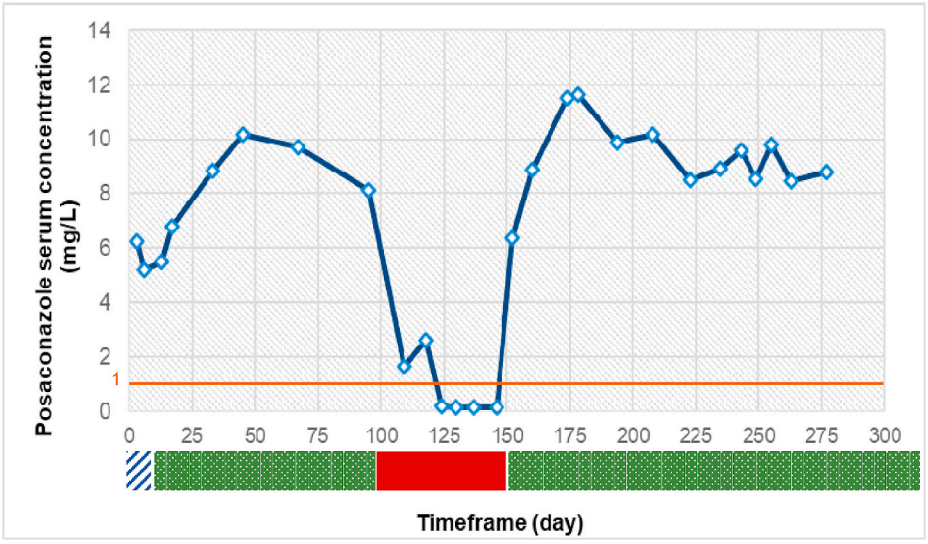

Fig. 2. Posaconazole serum concentrations. Orange line: minimum inhibitory concentration (MIC) of the mucormycete isolated. Striped blue rectangle: 5 mg/kg, PO, BID posaconazole regimen (day 0–8). Dotted green rectangles: 10 mg/kg, PO, SID posaconazole regimen (day 9–95 and day 150–405). Filled red rectangle: period where posaconazole was discontinued.

the two drugs [7].

Since no literature support was known regarding the use of delayed-release tablets of posaconazole in dolphins, the regimen used was the same reported for the posaconazole oral suspension – 5 mg/kg, PO, BID [8]. Tablets were chosen over the latter not only because of the tablets improved exposure described in humans [9], but also because compliance would be challenging since high volumes of the oral suspension would need to be placed into small pieces of fish, as the calf was still suckling and so, on a mainly milk-based diet.

Medical management of this case was based on a thorough follow-up, especially regarding the correction of the adverse clinical signs associated with the antifungal therapy, such as nausea and anorexia. Nausea has been described associated with posaconazole therapy in humans [10] but, to the authors' knowledge, there is no published information on adverse reactions to this drug in cetaceans. The change of the posaconazole regimen from 5 mg/kg, PO, BID to 10 mg/kg, PO, SID appeared to be a valuable and effective alternative to reduce adverse effects.

In humans, a mild hepatic enzyme level elevation associated with posaconazole therapy is described [10]. Similarly, hepatic evaluation throughout this clinical case only showed slight increases in transaminase levels, therefore it is believed that clinical hepatotoxicity was not one of the adverse effects related to the posaconazole therapy.

The optimal frequency for monitoring serum concentrations throughout posaconazole therapy is unknown and consideration of the clinical circumstances should guide the frequency of measurements [11]. In this study, however, posaconazole concentrations were not evaluated throughout treatment and were only studied retrospectively. The choice of samples was made according to the calf's clinical status in specific periods.

Results showed higher serum concentrations (reaching 11.63 mg/L) compared to the successful reports of mucormycosis management in dolphins where posaconazole concentrations were monitored [12,13]. It is important to note, however, that in these cases a different formulation (oral suspension vs. delayed-release tablets) and a different regimen were used (5 mg/kg, PO, BID vs. 10 mg/kg, PO, SID). Although additional studies are needed to help explain the striking set of posaconazole serum concentrations achieved, there are some aspects to take into consideration. Firstly, in humans, delayed-release tablets have shown improved pharmacokinetics compared to the oral suspension and therefore higher and more stable concentrations [9]. Moreover, while some studies show that tablets can be administered regardless of meals [9], in this case the ingestion of a fatty diet might have improved absorption of posaconazole. This is especially important when considering that dolphins' milk has a high-fat content, in this case 20%, and lactation was the primary source of nutrition of this calf.

In human studies, posaconazole serum concentrations seem to reach a steady-state around 6 days after beginning therapy, increasing in the first week and plateauing thereafter [14]. In this case, serum samples from days 3, 6, 13 and 17 showed posaconazole levels between 5.18 and 6.78 mg/L, and all later results from this first course of therapy showed concentrations above 8 mg/L. This suggests that a steady-state in this calf was also not obtained during the first week of therapy.

Although during the first period of posaconazole therapy, serum concentrations reached levels above 8 mg/L, a decrease was registered after initiating therapy (5.18 mg/L; day 6), which may be explained by a rapid distribution of the drug to fatty deposits, especially given its lipophilicity.

Overall, after resuming posaconazole therapy, there were slight changes in serum levels, possibly explained by small weight changes, occasional episodes of diarrhoea and possible variations in milk intake.

Although the area under the curve/minimum inhibitory concentration ratio (AUC/MIC) is the parameter best associated with the clinical success of posaconazole therapy [15], in this case it was not possible to calculate the area under the curve. A pilot study suggests that an AUC/MIC target of >100 may be a benchmark for future explorations of breakpoints in mucormycetes [16]. However, to measure this parameter, several blood collections (and involuntary procedures) would be necessary to get a set of results within a period of 24 h. For ethical reasons, this could not be taken into consideration. Given the above, the results of the posaconazole drug monitoring were compared to the MIC of *C. bertholletiae* (1.0 mg/L), although this does not incorporate pharmacokinetic parameters.

In this case, and only taking into consideration the steady-state, concentrations achieved were, for a long period, 8 to 11 times the MIC of the pathogen isolated. Although disputable, this may support the use of delayed-release tablets of posaconazole (10 mg/kg, PO, SID) in future cases of mucormycosis in bottlenose dolphins. Further species-specific pharmacological studies would be needed to explore this therapeutic option.

Given the set of results achieved, the dosage used could eventually have been reduced, possibly mitigating the adverse effects described.

However, clinical decisions on this subject are challenging given the known controversies of serum concentration targets [17].

Several theories can be suggested regarding the relapse of the fungal infection. As previously discussed, given the overall high results of the posaconazole concentrations and despite the lack of target drug levels, subtherapeutic serum concentrations may not be a plausible cause for disease reactivation. Notwithstanding the short length of the first course of therapy (96 days), perhaps the main explanation for relapse resides in the capacity of mucormycetes to invade tracheal cartilage, where posaconazole may not entirely reach given its high lipophilicity, independently of the overall high serum concentrations. Accordingly, although there were no signs of infection in the diagnostic routine tools, including bronchoscopy, a reservoir of organisms might have been present in the cartilage. Moreover, mucormycetes seem to be able to develop an intracellular lifestyle within granulomas, which can lead to the possibility of relapses [18].

Some specificities needed to be taken into consideration in the presented case since the animal was a calf. These included the fact that this dolphin was still in the learning process of several voluntary medical behaviours, and therefore most medical procedures revealed to be challenging. In addition, since milk was an important component of the calf's diet, compliance with oral drug therapy needed to be considered. Early fish introduction to the calf proved to be crucial to guarantee medication and thus avoid further involuntary procedures.

Since only one individual was considered, conclusions from this case may be biased and the authors reason that a continuous follow-up is still needed to investigate long-term disease reactivation. Discussion of results is quite challenging, especially given the lack of specifically described reported cases worldwide and the many clinical, pharmacological and microbiological controversies that still exist in the approach of mucormycosis.

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There are none.

Consent

Written informed consent was obtained from the patient or legal guardian(s) for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal on request.

Declaration of competing interest

There are none.

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