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Multiple Organ Dysfunction Syndrome (MODS) induced by *Candida krusei* in an Aldabra giant tortoise (*Aldabrachelys gigantea)* and confirmed by electron microscopy analysis

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ABSTRACT

A young female Aldabra giant tortoise (*Adabrachelys gigantea*) was presented with anorexia, ataxia, severe constipation and bloating. Analysis revealed liver disease and collected biopsy diagnosed *Candida krusei* infection. Despite Itraconazole treatment, the tortoise got worse and died. Full necropsy was performed; microbiology showed *Candida krusei* presence in liver, but histopathology didn't confirm fungal presence with special stains, so scanning electron microscopy was essential to prove a detailed diagnosis of extensive mycosis.

1. Introduction

Aldabra giant tortoise is an endangered species classified as vulnerable in the IUCN (International Union for Conservation of Nature) Red list of threatened species [1]. Studies on the ecology, environmental conditions, nutrition, health management and pathology of this species are essential in order to contribute to wildlife conservation in its natural habitats and captivity. Investigations carried out on sick or died animals held in zoological collections may prove significant to gain a better understanding of their diseases and potentially for therapeutic strategies since reports on the infectious disease in chelonians are still few in the literature.

Candida krusei has long been considered to be a transient commensal in man, and is an emerging fungal nosocomial pathogen primarily found in immunocompromised patients and those with hematologic-oncologic malignancies. In veterinary medicine it is often associated with mycotic mastitis [2,3], it has been described as responsible for necrotizing ventriculitis in parrots [4] and it has also been isolated in faecal samples of wild birds [5]. In reptiles *Candida krusei* was identified in tortoises [6] but was not regarded as a pathogen; using scanning electron microscopy here we describe the pathological characteristics of *Candida krusei* driven infection in an Aldabra giant tortoise; to our knowledge this is the first report on systemic candidiasis in an Aldabra giant tortoise.

2. Case

A 4 years old female Aldabra giant tortoise (*Aldabrachelys gigantea*) was presented in early November 2015 at day 0 for anorexia, ataxia and nasal mucus streaming. During physical examination microbiological swabs from the mucus and oral cavity and blood sample were collected and X-ray analysis was performed. Aerobic cultures isolated growth of *Escherichia coli* and radiographic evaluation showed the presence of a severe constipation and bloating. Blood work revealed high levels of Aspartate aminotransferase (AST) and Lactate dehydrogenase (LDH) liver enzymes, suggesting hepatic disease although the interpretation of liver function using only blood parameters is not well described in reptiles [7].

Considering the importance of the oral medications to treat constipation and patients' reluctance to be handled, oesophagostomy was performed on day 10 in order to place a gastric tube [8]. The adopted anesthesiological procedure was as follows: Ketamine (10 mg/kg IM) (Lobotor®, ACME, 42025 Cavriago, (RE) Italy) as pre-anaesthetic and after 45 min propofol (8–10 mg/kg per IV) (Propofol IBI*5F 20 ml 10 mg/ml, Ibi Lorenzini, 04011 Aprilia Latina (LT), Italy) using a 22 G intravenous catheter inserted into the jugular vein. Surgical approach

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was used for the application of the gastric tube: lateral incision of the skin of the neck was operated permitting the oesophagostomy and the lateral insertion of the catheter through the oesophagus into the stomach [9]. Fluid therapy was prescribed to rehydrate the animal and the intestinal mass: a continuous tube infusion of Ringer (2.5 ml/Kg/h) (SALF S.pA. Laboratorio farmacologico, 24069 Cenate Sotto (BG) Italy), which had been adjusted to 5% glucose (Baxter S.p.A. 00196 Roma, Italy), and some vaseline oil (1 ml/Kg/h) (Marco Viti Farmaceutici S.P.A, 22076 Mozzate (CO) Italy), was provided in order to soften the faecal impaction and to facilitate its intestinal transit. In this regard, we used some B-Braun infusion pumps (Infusomat fmS), thus helping to administer large volumes per diem and avoid any risks of regurgitation.

Moreover, enrofloxacin (5 mg/kg sid per OS for 15 days) (Baytril 25 mg/ml, Bayer Animal Health, 20121 Milan, Italy), calcium gluconate (100 mg/kg sid IM for 5 times) (Calcio PH*Iniet fl 500 ml, Fatro SpA, 40064 Ozzano Emilia, Italy) and Adecon multivitamin (2 ml IM once a week for 3 times) (Adecon *Im Fl 50 ml, Fatro SpA, 40064 Ozzano Emilia, Italy) were administered.

After 3 days of treatment the patient showed some signs of recovery and during the following days it began to expel some excrements. The radiographic examinations carried out at days + 17, + 24 and + 31 confirmed the disintegration of the faecal impaction. Even though the animal showed a significant improvement of the general condition it never started to eat again and at day + 50 we decided to perform a laparoscopic endoscopy under general anaesthesia. A considerable liver marbling was observed then swab and liver biopsy were collected for further investigations.

The sample collected by means of sterile cotton swab was seeded on Columbia Agar with 5% sheep blood (BD Becton Dickinson GmbH, Germany), CHROMagar, MacConkey Agar and Sabouraud with 0,05% chloramphenicol (CAF) for microbiological analysis; the plates were incubated at 25 °C in aerobic atmosphere until a noticeable growth was observed.

Portions of tissue from the liver were fixed in buffered formalin and routinely processed for histopathology. Four-micrometer serial sections from paraffin-embedded material were submitted to Hematoxylin and Eosin (H-E) and Periodic Acid Schiff (PAS) stains.

Blood agar, CHROMagar and MacConkey did not show any bacterial growth, while cream colonies were isolated on Sabouraud with CAF as pure culture, suggesting yeasts, starting from day 2 post incubation. A smear made from colonies taken from Sabouraud revealed a “long grain rice” appearance of the cells on microscopy suggesting *Candida krusei*. To definitely identify the fungal isolate, *Candida* species was identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF). The sample was prepared according to the manufacturer's instructions. Briefly, two to three yeast colonies were transferred using a 10-μL inoculating loop into 300 μL of distilled water and 900 μL of ethanol (Sigma-Aldrich, St. Louis, MO, USA). The suspension was pelleted after centrifugation at 6000*g* for 3 min, dried, and reconstituted in 50 μL of 70% formic acid (Sigma-Aldrich). After incubation for 30 min, 50 μL of acetonitrile (Sigma-Aldrich) was added. The suspension was then centrifuged at 6000*g* for 3 min. A volume of 1.5 μL of the supernatant was applied to a 96-spot Anchorchip™ target (Bruker Daltonics, Inc., Bremen, Germany) plate and dried. A saturated solution of 1.8 μL of MALDI matrix (HCCA; Bruker Daltonics, Inc., Bremen, Germany) was applied to the fungal smear and dried. Measurements were performed with a Microflex™ mass spectrometer (Bruker Daltonics, Wissembourg, France) using the FlexControl™ software (version 3.3.108.0). Mass spectra ranging from 2000 to 20,000 Da were acquired in a linear, extraction mode with positive polarity. The spectrum was imported into the Biotyper software (version 3.0; Bruker, Karlsruhe, Germany). The generated spectrum of biomarkers for each sample was then compared with reference spectra in the Bruker library. Identifications from MALDI-TOF were classified using modified score values proposed by the manufacturer: a score ≥ 2 indicated identification to the species level; a score between 1.7 and 1.99 indicated identification to the genus level; and a score of < 1.7 indicated no identification. The score value of the isolate was 2.321.

Histopathological investigations confirmed the presence of multiple chronic granulomas but didn’t demonstrate fungal or bacterial presence despite H-E and PAS stains.

At day + 72 unfortunately the animal died despite treatment with itraconazole (5 mg/kg sid per OS for 21 days) (Itraconazolo Teva 100 mg, Teva Italia S.r.l., 20123 Milan, Italy) and full necropsy was performed.

Necropsy showed a serious cachexia. The liver size was increased and granulomas of variable dimensions from 1 to 5 mm were scattered on the surface of all lobes. In cutting section they had different depth and some of them were rasping. The kidneys were increased in volume and they had a pale pink color. The spleen showed rounded margins and it was bigger than normal size for this species. The heart macroscopically did not exhibit abnormalities except a diffuse pallor. The gastrointestinal tract, didn’t show abnormalities and presence.of parasites. The central nervous system and the respiratory tract didn’t show abnormalities.

During the necropsy microbiological swabs were collected from the liver, spleen and kidneys and multiple samples were collected from all organs and processed using histopathology and scanning electron microscopy (SEM) [10,11].

Molecular biology was also performed on liver samples testing mycobacteria.

Microbiology revealed *Candida krusei* in kidneys and liver and Polymerase Chain Reaction (PCR) was negative for *Mycobacterium spp*.

Histological examination demonstrated a severe inflammatory response in liver, spleen and kidneys where organ architecture was altered including necrotic areas, abundant fixed scar tissue and granulomatous lesions.

In the liver localized accumulation of heterophils and macrophages (heterophilic abscess) were observed. Granulomas were also present in the kidney, varying in size, and microscopically they appeared as multifocal coalescing lesions containing a central mass of eosinophilic, granular cellular debris surrounded by a diffuse zone of heterophils, macrophages and multinucleated cells. Granulomas with a mineralized core occurred in large amount suggesting a prolonged inflammatory response.

Ziehl-Neelsen, Fite-Faraco, PAS and Grocott-Gomorri special histological stains didn’t demonstrate mycotic or bacterial elements.

To perform SEM the specimens were divided in two different groups and prepared in accordance with the protocol described as follows and named “Osmic maceration”: after an initial reduction in size of few mm [3], the specimens were washed in phosphate buffered saline (PBS, pH 7.2) and then post-fixed in a solution of 1% osmium tetroxide and 1.25% potassium ferrocyanide for 2 h. Specimens were further reduced in slices 1 mm thick, followed by a second post-fixation in 1% osmium tetroxide and 1.25% potassium ferrocyanide for 1 h. The slices were washed in PBS and immersed in 0.1% osmium tetroxide in PBS for 48 h [12]. They were then dehydrated in graded ethanol and subjected to critical point drying with CO_2. The slices, mounted on aluminium stubs, were coated with 10 nm of pure gold in an Emitech K250 sputter-coater. Scanning electron microscopy (SEM) enabled the authors to observe in details the lesions produced by the fungus: in the liver a large number of both portal tracts (Fig. 1A) and sinusoids (Fig. 1C) appeared completely filled with fungal hyphae (Fig. 1B); furthermore some hepatic sinusoids displayed an extremely thickened wall exhibiting some structures morphologically compatible with bacteria (Fig. 1D).

Even in the vasculature large masses of fungal filaments were identified extensively invading blood vessels lumens (Fig. 2) and resulting in thrombosis. Scanning electron microscopy illustrated also that in the kidney the parenchymal structure was completely subverted and showed many scattered calcified deposits conferring a granular texture to the tissue (Fig. 3).

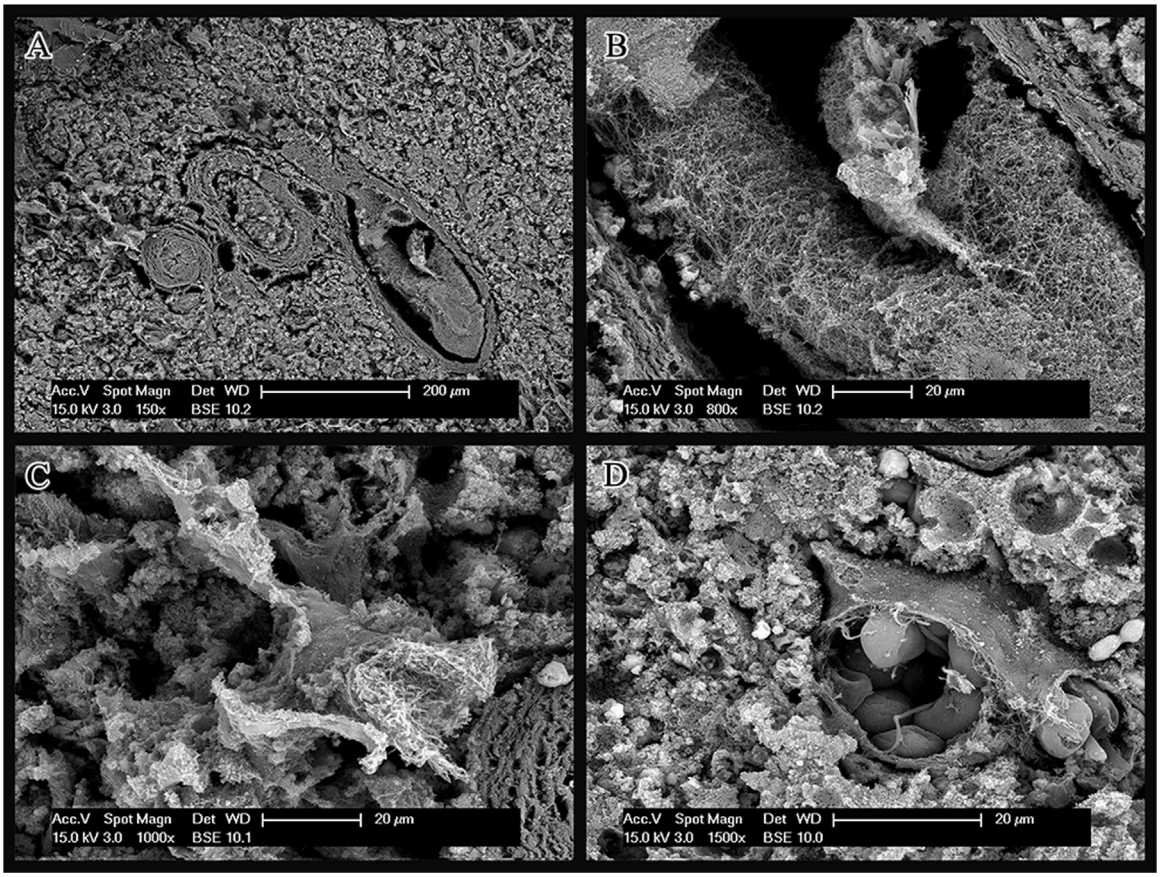

Fig. 1. 1A. Section of liver parenchyma: the vascular structures are totally obstructed. 1B. Detail of previous image: the large blood vessel is completely full of fungal hyphae, you do not see the presence of any red blood cell, even fragmented. 1 C. Plexus sinusoidal completely packed with fungal hyphae. 1D. In this detail it is possible to see a vessel with few red blood cells morphologically preserved mixed with sporadic fungal hyphae.

3. Discussion

The present report firstly describe a Multiple Organ Dysfunction Syndrome (MODS) starting from *Candida krusei* liver infection in an Aldabra giant tortoise. In reptiles liver fungal diseases are not yet well described even if systemic mycoses are very common in herpetological medicine [13–17]; in particular *Candida krusei* was identified in tortoises [6] but was not regarded as a pathogen.

Given the importance of the patient belonging to endangered species list, investigations were carried out to better understand the specific pathogenicity of fungal disease in tortoises. In fact it seems that mycotic diseases are underestimated in current reptile medicine; habitat humidity and diet characteristics are often considered the responsible cause of fungal presence in oral cavity and gastrointestinal tract of these animals. For this reason literature is lacking in this subject [18] that deserves further studies. The authors started the fungal diagnostic workflow with culture of swabs from the liver which isolated growth of *Candida spp*. colonies later speciated with MALDI-TOF mass spectrometry and identified as *Candida krusei*; concurrently the authors investigated the presence of pathogens in the liver by histology, but they could not demonstrate the presence of bacteria or fungi in the tissue. Among the possible reasons for the discrepancy existing between the microbiology and the histology is the possible low fungal load; it has also to be stressed that, unfortunately, the private laboratory performed the histological analysis of biopsy sample by standard stains H-E and Periodic acid-Schiff (PAS) and failed to study in depth the possible presence of fungi by using the Grocott-Gomorri silver stain that is more sensitive for fungal elements than the PAS one.

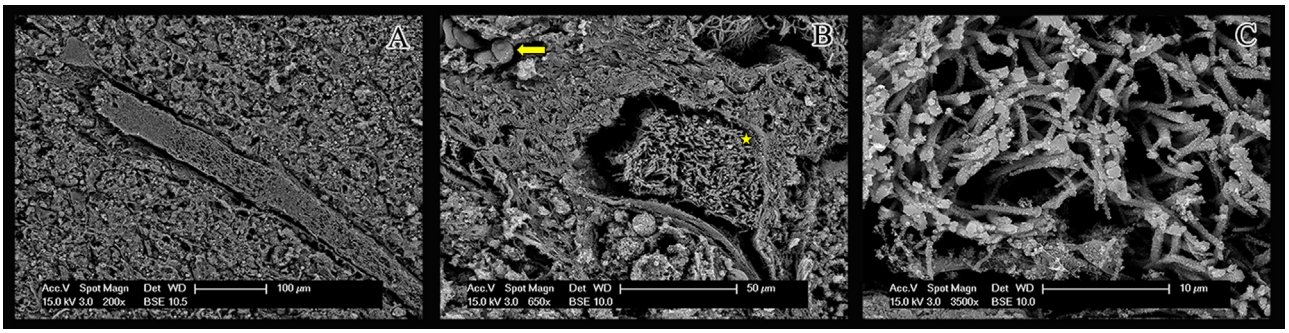

Fig. 2. 2A Vascular structures full of fungal hyphae. 2B Example of lumen of hepatic sinusoid (star) densely packed with fungal hyphae and lumen containing some red blood cells with preserved morphological structure (arrow) and sporadic hyphae. 2 C. Details at higher magnification of hyphae present in the vessel of Fig. 2B.

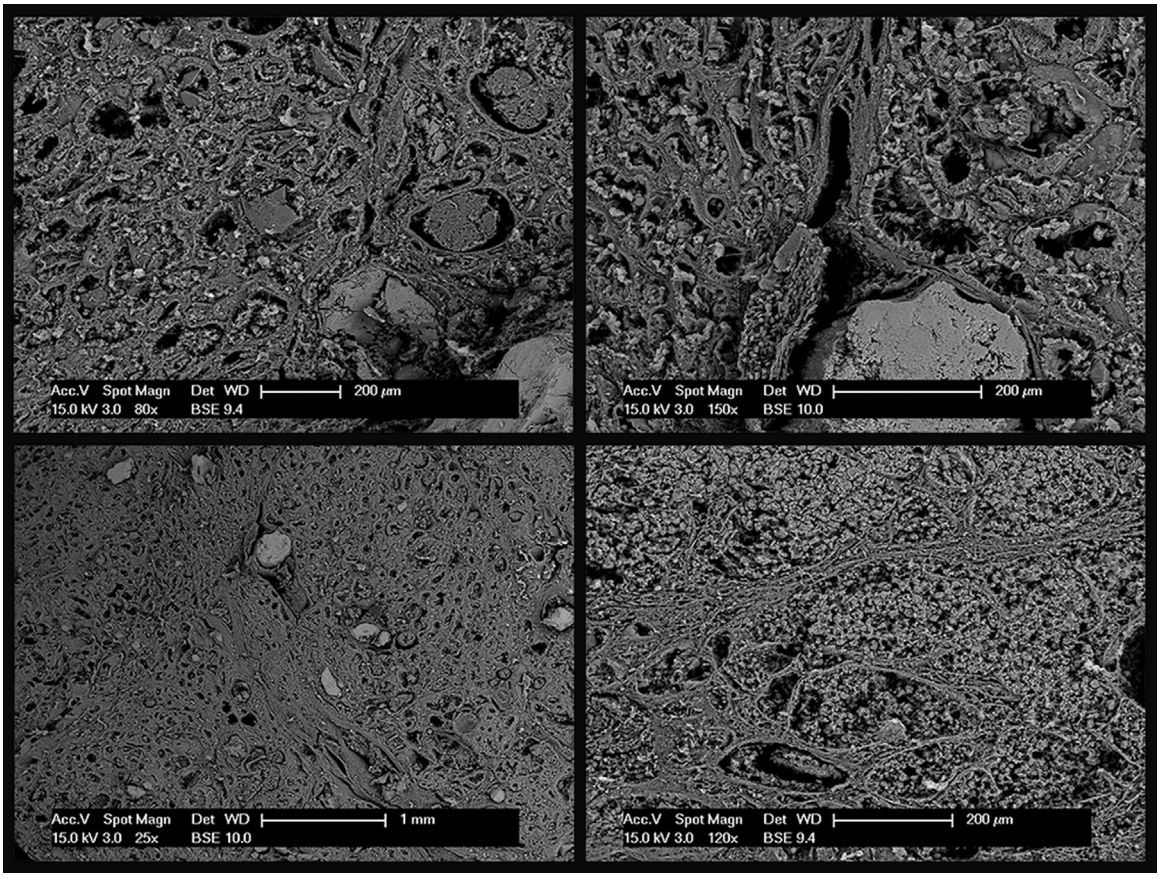

Fig. 3. A-B-C. Kidney parenchyma where it is possible to see many calcified deposits. D. The area of greatest structural subversion was observed close to at the hilar zone (where the lobe is connected with the main axis of the collector of single lobe).

Despite discrepancy the authors were confident that the most direct and conclusive mean of establishing the diagnosis of the fungal infection was the growth of the fungus from the patient samples and started itraconazole treatment of the tortoise.

At necropsy the diagnostic workup revealed *Candida krusei* positive cultures of samples collected from the kidney that combined with the previous results obtained from the liver suggested a progressive fungal invasion of tissues and vessels; histopathologic examination of specimens performed with H-E, PAS, and the special Grocott-Gomorri staining of the tissue, as fungi were suspected, didn't confirm fungal presence. On the other hand the scanning electron microscopy (SEM) showed the presence of pathogenic microorganisms-fungi and bacteria-in the liver, vasculature, and kidney and was effective to define the extent of fungal invasion of tissues and blood vessels where fungal filaments built up stenosis responsible, at least in part, for the animal death despite antifungal treatment. Moreover the SEM technique allowed a detailed visualization of liver, vascular, and kidney tissue architecture with their abnormalities, thus strengthening the results obtained by histological examinations.

To give an explanation for the discrepancies between the histopathology and SEM results the authors hypothesized that tissues were sampled from two different areas and that the one containing viable fungi was sent to microbiology while the second sample, not containing the fungal elements, was sent to pathology.

It also interesting to point out that comparing the results obtained at biopsy with those collected at necropsy the authors cannot exclude the possibility that significant fungal proliferation occurred between the two time points despite itraconazole treatment. Another important aspect to consider is that more pharmacological studies need to be performed in veterinary medicine concerning antimycotic agents; azoles and in particular voriconazole are first choice antimycotic agents for treating invasive *Candida spp*. infection in human beings; triazole antifungals have been reported in the literature also for the treatment of mycoses in reptiles, but due to data lack about voriconazole safety and efficacy in chelonians, in the present case the tortoise received itraconazole.

Indeed in veterinary medicine scanning electron microscopy is not routinely used as diagnostic tool because of costs and difficulty of specimen preparation but it could be an effective investigation technique because SEM results interpretation provides valuable morphological and structural informations related to organs and tissues supplying data on the growth and colonization by microorganisms that it is impossible to define with other diagnostic methods.

In conclusion the authors report a *Candida krusei* driven systemic mycosis which led to a Multiple Organ Dysfunction Syndrome (MODS) in an Aldabra giant tortoise and demonstrate that the use of innovative techniques of detection of pathogenic microorganisms such as scanning electron microscopy can be combined with the traditional microbiological methods and may represent a valuable tool in order to help to formulate detailed diagnosis as these techniques are able not only to provide informations about the pathogen, but also to quantify the damage produced by the pathogen and also to clarify the efficacy or inefficacy of a drug treatment.

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Conflict of interest

There are none conflict of interest.

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