Subcutaneous phaeohyphomycosis due to Aureobasidium pullulans *infection in a dog*

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Aureobasidium pullulans,
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Differential diagnosis,
Dog,
Opportunistic infection.

Summary
A case of subcutaneous phaeohyphomycosis in a dog on the right limb during a post-operative period of castration was described for the first time. The macroscopic and microscopic characteristics of the fungal colonies growth on the Sabouraud-dextrose agar were detailed. The fungus was identified as *Aureobasidium pullulans* on the basis of phenotypic analysis and confirmed by sequencing of the internal transcribed spacers (ITS) region of rDNA. The spontaneous remission of the lesion was observed in five weeks without antifungal treatment. This work highlights the importance of considering the pathogenic potential of this environmental fungus and the need of including it in the differential diagnosis of cutaneous lesions in dogs.

Introduction

Phaeohyphomycosis is an opportunistic infection caused by dematiaceous fungi, which include the *Aureobasidium* genus. This fungal genus recognised as uncommon pathogen, is now known as hospital contaminant and emergent pathogen (Chan *et al.* 2011). Most of the infections in humans and animals occur by the traumatic implantation from environment soil and plants (Lloret *et al.* 2013, Wang *et al.* 2019).

Aureobasidium pullulans is the main fungal species responsible for cutaneous (Chen *et al.* 2016, Pikazis *et al.* 2009) and subcutaneous (De Oliveira *et al.* 2013, Joshi *et al.* 2010) lesions in immunocompromised and immunosuppressed humans. This species also is reported causing systemic involvement, in particular at pulmonary level, difficult to differentiate from other important infections (Hofman *et al.* 2008).

In veterinary, the reports of *A. pullulans* infections are rare and include canine cases of otitis (Campbell *et al.* 2010) and disseminated infections (Perkins *et al.* 2004). Cases of cutaneous lesions in a porcupine (Salkin *et al.* 1976) and in a cat have also been observed (Bernhardt *et al.* 2015).

In this report we describe the first case of subcutaneous phaeohyphomycosis in a dog due to *Aureobasidium pullulans* infection.

Case report

A swab from a cutaneous lesion of a 5-year-old Labrador female dog, living in Rio Grande/RS (Southern Brazil), was sent to our laboratory with the clinical suspicion of sporotrichosis. The lesion was located on the femorotibial-patellar joint of the right posterior limb. It was a single, circumscribed exudative and ulcerative lesion with alopecic borders and dark-crust in the center, that, when removed, exposed a pinkish lesion with the blackened center (Figure 1A).

Anamnestic data revealed that the animal had access to the courtyard of the house and residual medication of a recent ovariohysterectomy. The lesion in the limb appeared during the postoperative period, and no other clinical alterations were noted.

Mycological analysis

The laboratory procedures were performed according to the solicited suspicious of

sporotrichosis. The direct examination of the sample did not show cigar-like cells in Gram staining but oval structures arranged in strings. The sample was sown in duplicate on Sabouraud-dextrose agar (SDA) with chloramphenicol and in Mycosel® agar. Both agars were incubated at 25 °C and 37 °C to confirm the dimorphic characteristic of *Sporothrix* sp.

After six days, smooth and white colonies with creamy appearance were observed on the SDA plates at both temperatures (Figure 2A). The microscopic analysis showed unicellular and budding yeast-like cells, and consequently the sporotrichosis suspicious was discarded.

The colonies were observed daily and, on the 10th day of incubation, they changed the smooth borders to an irregular appearance similar to a fringe and became dark, leading us to suspect infection by *Aureobasidium pullulans*. The direct microscopic examination of lactophenol cotton blue (LCB) mount showed numerous hyaline yeasts cells with some dematiaceous oval cells (Figure 2B).

On the 15th day, these cells changed to hyaline or dematiaceous delicate hyphae with thin-walled, producing small blastoconidia and directly from the walls at certain fertile points (Figure 2C). On the 19th day, the colonies were almost totally dark (Figure 2D) and the examination of LCB showed hyphae with thick-walled, dark, and septated segments with blastoconidia (Figure 2E).

Molecular analysis

This isolate was named as 'MV 2578' and was genetically characterized by sequencing the internal transcribed spacer (ITS) using the primers ITS1 (5'-TCC GTA GGT GAA CCT TGC GG) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC) (Irinyi *et al.* 2015). It was identified by data bank analysis with NCBI BLASTn (identity 99%) and the sequence was submitted online at Genbank number MG595273.1.

For phylogenetic analysis, the sequences of the isolated strain were compared with the homologous sequences of *A. pullulans* reference strains and clinic relevant isolates obtained from Genbank (Figure 3). The multiple sequences alignment obtained from the ISHAM ITS and Genbank databases showed 95% identity between *A. pullulans* MV2578 sequence ITS-4 and 15 human and veterinary clinical isolates. *A. pullulans* MV2578 sequence showed also 82.5% identity with AY4 and 2712H strains, and 75% with HN4.4 strain.

Follow-up of the procedures, and the risk factors

In case of suspicious of *Aureobasidium* sp., the direct examination should be performed with 20% potassium hydroxide solution in skin samples for the search of multiple thick-walled and dark hypha with oval cells containing dark septa (Chen *et al.* 2016, Larone 2011). Although the swab was processed

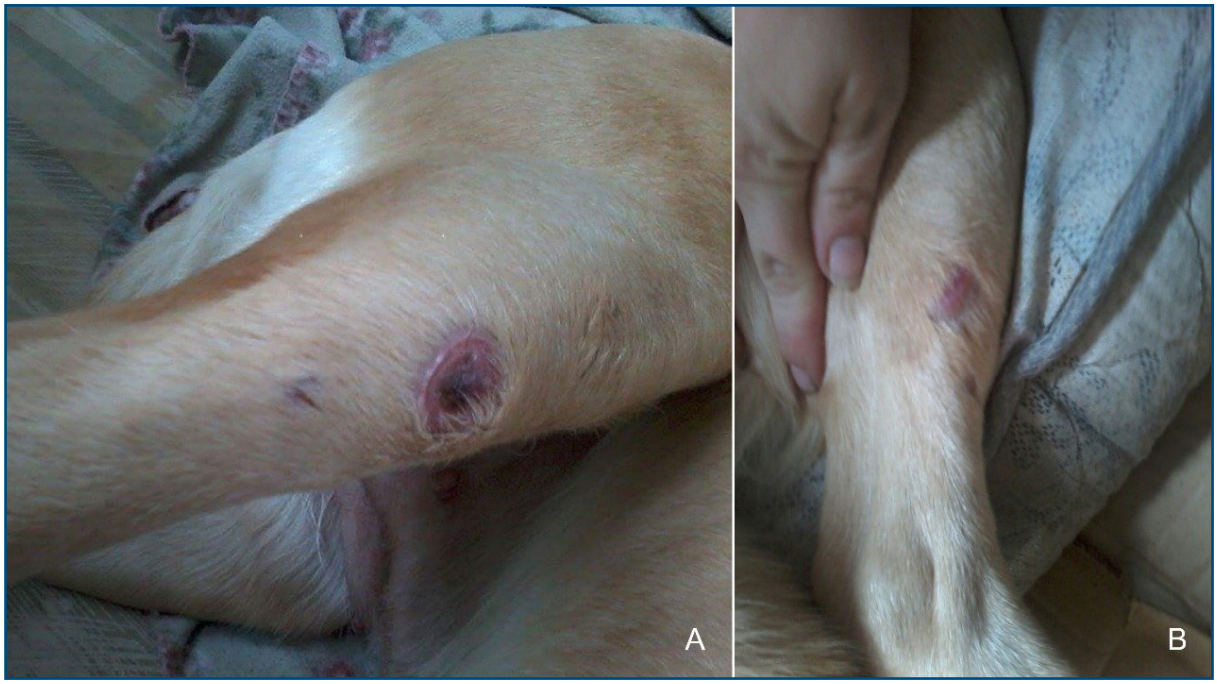

Figure 1. *Subcutaneous lesion located on femoro-tibial-patellar joint of the right posterior limb of an immunocompetent Labrador female dog with* Aureobasidium pullulans *infection.* Note alopecia on the borders of the exudative and ulcerative lesion and a dark-crust in the center (**A**), and the clinical spontaneous remission after five weeks (**B**).

according to the clinical suspicious solicited by the veterinarian, in the Gram staining we noted oval structures arranged in strings, which were compatible to the arrangement of fungal elements described by Chen and colleagues (Chen *et al.* 2016).

Aureobasidium pullulans was suspected when a dark fringe appeared on the border of colonies and the macromorphological and micromorphological characteristics of the fungal structures were similar to those described by Larone (Larone 2011). The diagnosis of phaeohyphomycosis is generally based on fungal detection by cytology and/or histology. Cultures however provide definitive diagnosis and species identification (Lloret *et al.* 2013). The diagnosis of *A. pullulans* relies on direct microscopic examination of clinical samples which may be misleading and should be confirmed by the molecular assay (Chan *et al.* 2011). In our case, the fungal species was confirmed through the ITS sequence polymorphism.

According to the anamnaestic data, the subcutaneous lesion on the right posterior limb appeared after castration, although the surgical scar did not show cutaneous alterations. However, postoperative stress might have played an important role. It is known that the ovariohysterectomy deregulates B and T cells (Nenadović *et al.* 2017), affecting the immune system. The blood loss and the tissues trauma due to the surgery also stimulate proinflammatory (IL-6 and TNF-α) and anti-inflammatory (IL-10 and TGF-β) cytokines which block the cell-mediated immune responses (Angele and Faist 2002) and increase the susceptibility of the host to infection.

It is believed that a traumatic inoculation related to the environmental contact (Chen *et al.* 2016, Lloret *et al.* 2013, Pikazis *et al.* 2009, Wang *et al.* 2019) was the source of infection, since the animal had free access to the courtyard of the house. Considering that *Aureobasidium pullulans* is a black-yeast-like surface colonizer and is commonly associated as contaminant in hospital (Wang *et al.* 2019), it is important to be aware of the possible risk of false positives in the diagnosis. A histopathological analysis is recommended to demonstrate tissue invasion (Perkins *et al.* 2004). In this case, however, it was not possible to perform histopathology because the owner did not allow the animal to undergo a new surgical procedure.

Various agents of subcutaneous or systemic phaeohyphomycosis are similar in appearance and,

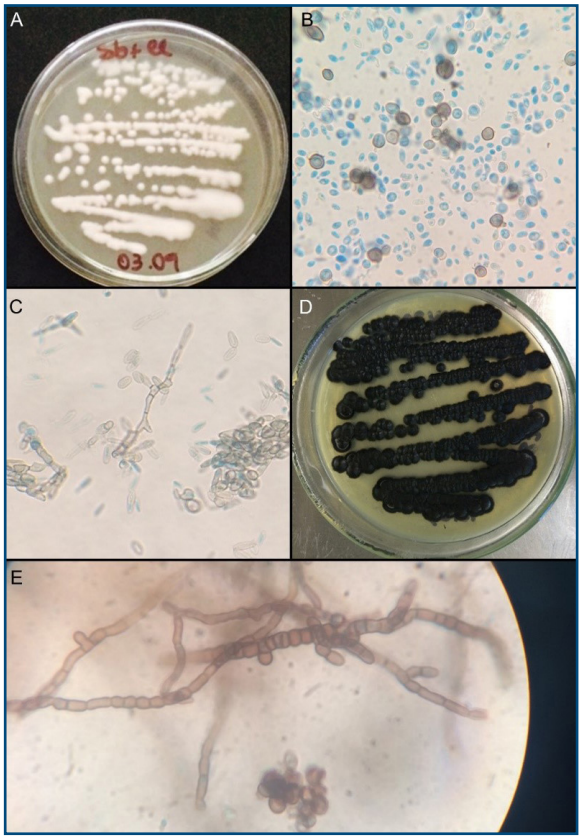

Figure 2. Aureobasidium pullulans *MV 2578 (Mycology Collection of Centro de Diagnóstico e Pesquisa em Micologia Veterinária, UFPEL, Brazil).* **A.** Macroscopic appearance on Sabouraud-dextrose agar after the 6 day of incubation at 37 ºC and microscopic characteristic using Lactophenol Cotton Blue, note hyaline and dematiaceous oval cells (**B**, 1000×); on the 15th day, dematiaceous hyphae with fertile points were noted (**C**, 1000×); the colony appearance changed to dark (**D**) after 19 days with hyphae showing thick-walled and septated segments with blastoconidia (**E**, 1000×).

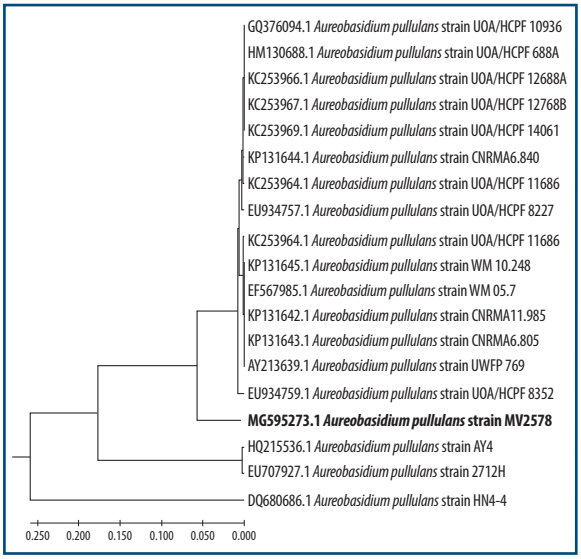

Figure 3. *Neighbor-joining phylogenic analysis of ITS of* Aureobasidium pullulans *MV 2578 and most significant culture collection strains and clinically relevant isolates.* The optimal tree with the sum of branch length = 0.77464334 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method (Irinyi *et al.* 2015) and are in the units of the number of transitional substitutions per site. There was a total of 285 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 software (Tamura *et al.* 2004).

frequentlyand can't be differentiated by morphology. Cultures are essential to diagnosis. In our case, the culture allowed to the fungal identification, which was confirmed by the molecular analysis. Still, the growth of *A. pullulans* on the SDA on the agar plates and the absence of contaminants confirmed the high technical skill of the operators.

Furthermore, due to the ability of this species to survive uncommon environments, the reuse of laboratory solutions and buffers for cytology (Hofman *et al.* 2008) must be avoided. All procedures in our case were individually performed with no risk of pathogen transfer to stock solutions, and this laboratorial care should be taken into consideration for all laboratories in any microbial suspicion.

The clinical outcome

After the sample collection, the veterinarian recommended daily topical applications of rifamycin. This therapy was interrupted after a week, due to the negative result of the bacteriological analysis. Once *A. pullulans* infection was confirmed, a protocol including oral itraconazole for four weeks was recommended in accordance with what has been described in the literature (Joshi *et al.* 2010, Pikazis *et al.* 2009 Chen *et al.* 2016, De Oliveira *et al.* 2013). However, due to financial matters, the owner decided not to treat the animal. *Aureobasidium pullulans* was repeatedly isolated even if the lesion started to decrease spontaneously in a few days. In five weeks, the lesion recovered, showing that antifungal therapy was not critical in this case.

Furthermore, some general practices are also important, such as keep clean the body site with physiological solution many times a day, avoid contaminated environment, keep the patient well fed, and avoid any aggravating stressful situation.

Conclusions

This is the first report of subcutaneous phaeohyphomycosis in a dog caused by *Aureobasidium pullulans*. This case report stresses the importance of including *A. pullulans* as a possible fungal pathogen in dogs when ulcerative lesions in subcutaneous tissue are observed, and, more in general, the importance of considering infections by unusual fungi as opportunistic infections in the diagnosis of cutaneous lesions.

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