Scedosporium apiospermum isolated from a skin wound in a dog and in vitro activity of eleven antifungal drugs

Scedosporium apiospermum *geïsoleerd uit een huidwond bij een hond en in-vitroactiviteit van elf antischimmelmiddelen*

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ABSTRACT

***Scedosporium apiospermum*, an emerging opportunistic fungus, is increasingly implicated with serious invasive mycoses in immunocompromised patients. Rare occurrence and difficulty in identification of this species complicate the management of infection in dogs. In the present report, the characterization of a *Scedosporium apiospermum* isolate obtained from a dog's skin wound is described. Identification was obtained through macroscopic and microscopic examination of the cultured isolate and by sequencing the internal transcribed spacer (ITS) gene of the rRNA region. Numerous hyaline septate hyphae along with conidia were observed histologically. The isolate exhibited low MIC values for voriconazole, itraconazole and isavuconazole while high MIC/MEC values were recorded for the remaining antifungals. To the best of the authors' knowledge, this is the first reported case of isolation of *S. apiospermum* from a skin wound in a dog.**

SAMENVATTING

Scedosporiumapiospermum, een opkomende opportunistische schimmel, wordt in toenemende mate gezien bij ernstige invasieve mycosen bij immuungecompromitteerde patiënten. Door het zeldzame voorkomen en de moeilijkheid om deze soort te identificeren, is de behandeling van infectie bij honden niet evident. In deze casuïstiek wordt het karakteriseren van een *Scedosporiumapiospermum*-isolaat verkregen uit een huidwond van een hond beschreven. De identificatie werd verkregen door macroscopisch en microscopisch onderzoek van het isolaat en door de sequentie van het interne overgeschreven spacer (ITS)-gen van het rRNA-gebied. Talrijke hyaline-septaathyfenenconidia werden histologisch waargenomen. Het isolaat vertoonde lagere MIC-waarden voor voriconazol, itraconazol en isavuconazol, terwijl hogere MIC/MEC-waarden werden geregistreerd voor de overige antischimmelmiddelen. Voor zover de auteurs bekend, is dit het eerste gerapporteerde geval van isolatie van *S. apiospermum* uit een huidwonde bij een hond.

INTRODUCTION

Scedosporiumapiospermum is a dematiaceous fungal pathogen found worldwide in soil, polluted waters, sewage, indoor plant pots and greenhouses (Signore et al., 2017). Although this fungus is rarely isolated, it can cause a wide spectrum of infections ranging from superficial, soft tissue invasions to disseminated infections in immunocompromised and immunocompetent hosts (Luplertlop et al., 2016). This leads to a variety of symptoms making the diagnosis difficult, and affects the treatment regime where an early initiation of medication is critical (Signore et al., 2017, Tan et al., 2020).

S. apiospermum shows intrinsic resistance against many antifungals, which is a topic of concern where commonly used antifungals are advised since it often progresses to fatal cerebral infections (Jalava-Karvinen

et al., 2016). The diagnosis of this pathogen is also complicated by clinical similarities with disseminated infections caused by *Aspergillus* spp. and *Fusarium* spp. in animals (Smith et al., 2018). Accurate diagnosis can be obtained by culture growth concurrently with molecular identification (Chen et al., 2016; Singh et al., 2020a).

The study was thus undertaken to phenotypically characterize, confirm by sequencing and investigate the in vitro profile of thirteen antifungal drugs against the *S. apiospermum* isolate obtained from a skin wound in a dog.

MATERIALS AND METHODS

Collection and processing of the sample

A dog (breed: pug) was presented with a history of dogfight, which had caused a bite wound on its right front leg. The dog was treated with topical Neosporin Skin Ointment® (Bacitracin-400IU, Neomycin-3400IU and Polymyxin B-5000IU) and advised for post-bite rabies vaccination. Ten days later, the same dog presented with swelling, pain and severe scratching in the same area. The wound was infected and the lesion started spreading to nearby areas and became dark in color (Figure 1). The owner told that the dog used to go swimming at a nearby lake on weekends. The area was disinfected with 70% ethanol to remove externally contaminating bacteria and fungi. About 0.5 ml fluid was aspirated with the help of sterile syringe from the wound. The sample was immediately transferred to the laboratory for further analysis. The sample was inoculated on Nutrient Agar (BD Difco™, India) at 37°C for 48 hours and Potato Dextrose Agar (PDA) containing 0.05% chloramphenicol (HiMedia®, India) at 35°C for ten days (Singh et al., 2020b). Isolated colonies were stained with Lactophenol cotton blue (LCB) stain to study hyphal structures and condial cells. Molecular confirmation was done by sequencing the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) with the help of primers ITS1 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC). Gene sequence was submitted to GenBank database under accession number MT895419. Tissue samples were sent for histological examination. Gomori Methenamine silver (GMS) staining was performed according to the protocol suggested by Grocott (1955).

Antifungal susceptibility testing

Antifungal susceptibility testing (AFST) was performed by Broth Micro-dilution Assay method recommended by Clinical Laboratory Standard Institute (CLSI) approved standard M38 (third edition) against eleven antifungals, namely amphotericin-B, flucytosine, fluconazole, ketoconazole, itraconazole

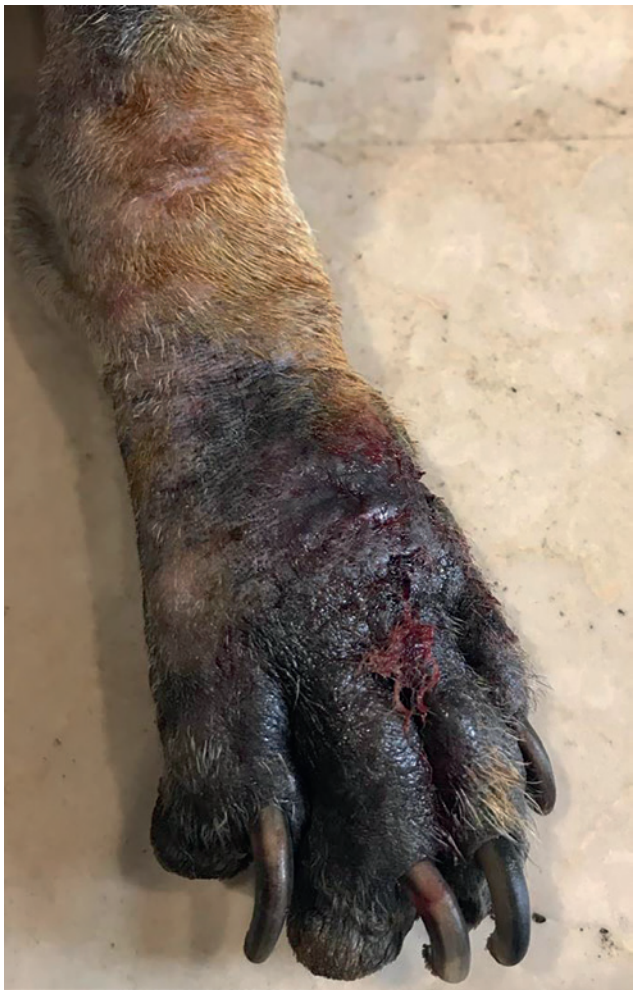

Figure 1. Deep swollen skin wound caused by *Scedosporiumapiospermum* in a dog (breed: pug).

Figure 2. Obverse section of *Scedosporium apiospermum* growth on Potato Dextrose Agar medium.

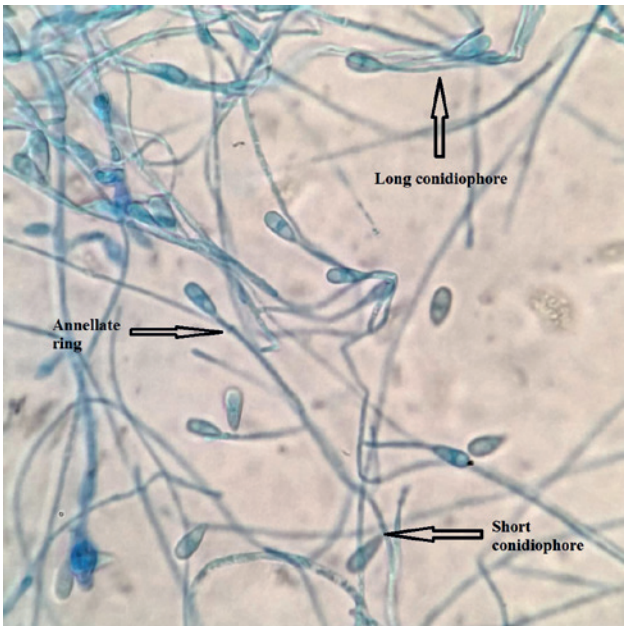

Figure 3. Lactophenol cotton blue staining showing hyaline and septate hyphae, annellate ring, short and long conidiophore under phase contrast microscope (100X).

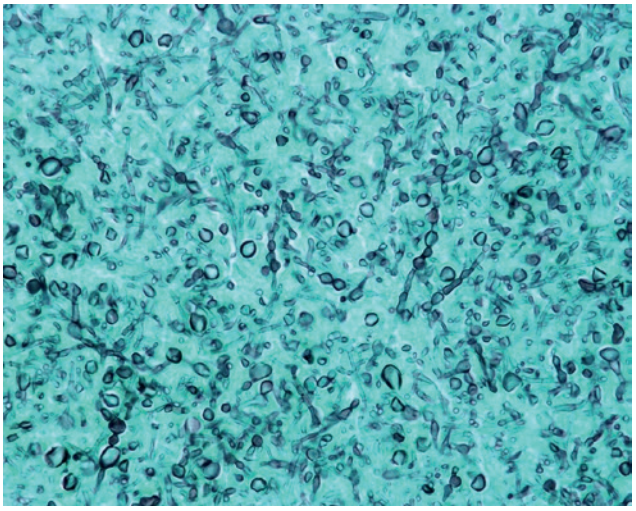

Figure 4. Gomori's Methenamine Silver (GMS) staining of skin wound showing numerous clusters of hyaline, branching, septate hyphae and conidia having a truncated base (400X).

voriconazole, posaconazole, isavuconazole, caspofungin, micafungin, and anidulafungin (all procured from Sigma-Aldrich®) (CLSI, 2017). In brief, all the drugs were dissolved using 100% dimethyl sulfoxide (DMSO, analytical grade) except fluconazole (in sterile water). Dilution was done in Roswell Park Memorial Institute (RPMI) 1640 medium containing l-glutamine (without sodium bicarbonate) and buffered at pH 7.0 with morpholinepropanesulfonic acid (MOPS) to obtain a final concentration of 0.0313 to 16 µg/ml for amphotericin-B, itraconazole, ketoconazole, posaconazole, voriconazole and isavuconazole; 0.125 to 64 µg/ml for flucytosine and fluconazole; and 0.008 to 4 µg/ml for caspofungin, micafungin and anidulafungin. The isolate could not be classified directly as susceptible to or resistant against these antifungals as clinical breakpoints are not well-defined against *S. apiospermum* (CLSI, 2017).

Fungal inoculum was prepared by taking the aerial hyphae from the colony and suspending in 1 ml of sterile 0.85% saline (CLSI, 2017; Singh et al., 2020b). Suspension was vortexed in a sterile tube, diluted with RPMI-1640 and cell density adjusted to 0.15 to 0.17 at 530 nm to acquire the final inoculum concentration of 0.4×10^4 to 5×10^4 CFU/ml. Column 1 of sterile micro dilution plates (Tarson®) was filled with 200 µl inoculum, column 2-12 filled with both inoculum and serially diluted antifungal agent (100 µl each) and finally incubated at 35°C without agitation. *S. apiospermum* (ATCC® 3635, HiMedia) was selected as a quality control strain in accordance with the criteria listed in the CLSI (2017) document. Minimum Inhibitory Concentration (MIC) was interpreted after 70 to 74 hours of incubation for all the antifungals except Echinocandins. For the Echinocandins, Minimum Effective Concentration (MEC) was used to improve reproducibility (read after 46 to 72 hours) (CLSI, 2017). The test was carried out in triplicates.

RESULTS AND DISCUSSION

At first, the macroscopic colonies on PDA were floccose white, which changed to brownish-black after production of aerial hyphae (Figure 2). Staining with lactophenol cotton blue revealed hyaline, septate hyphae. Short and long conidiophores were produced in small groups and at the point of attachment, annellate ring was observed which is typical for *S. apiospermum* (Baumgartner et al., 2007) (Figure 3). Basic Local Alignment Search Tool (BLAST) search of the sequencing result revealed 98.69% similarity with *Scedosporium apiospermum*. Histological examination revealed numerous black-colored fungal hyphae. GMS staining revealed hyaline, branching and septate hyphal structures and conidia having truncated base (Figure 4). These histological features are also consistent in other hyaline mycotic agents like *Aspergillus* spp. and *Fusarium* spp., which can result in delayed or inappropriate fungal treatment (Haynes et al., 2012; Smith et al., 2018). Due to these indistinct and overlapping features, most cases may remain undiagnosed or misdiagnosed as other mycoses. Therefore, for a definitive diagnosis of *S. apiospermum*, culture along with gene sequencing should be used as a gold standard.

S. apiospermum has been reported in dogs causing nasal granuloma, rhinitis, osteomyelitis, spondylitis, keratomycosis, pyelonephritis and myocarditis (Hugnet et al., 2009; Haynes et al., 2012; Matteucci et al., 2017). To the authors' knowledge, this is the first report of *S. apiospermum* isolation from a skin wound in a dog. The infection might have been established

Table 1. Minimum Inhibitory Concentration (MIC) of *Scedosporium apiospermum* isolate against thirteen antifungal drugs using broth micro-dilution assay (CLSI M38).

Fungus	Antifungal drug Concentration (µg/ml	Final Antifungal time (Hours)	Incubation MIC/MEC	MIC/MEC (µg/ml)	MIC/MEC (µg/ml) *S. apiospermum* ATCC® 3635)
Scedosporium apiospermum	Amphotericin-B	0.0313 to 16	70 to 74	16	8.0
	Flucytosine	0.125 to 64	70 to 74	>64	>64
	Fluconazole	0.125 to 64	70 to 74	>64	>64
	Ketoconazole	0.0313 to 16	70 to 74	16	8.0
	Itraconazole	0.0313 to 16	70 to 74	1.0	0.5
	Voriconazole	0.0313 to 16	70 to 74	0.5	1.0
	Posaconazole	0.0313 to 16	70 to 74	4.0	2.0
	Isavuconazole	0.0313 to 16	70 to 74	2.0	2.0
	Caspofungin*	0.008 to 4	46 to 72	4.0	2.0
	Micafungin*	0.008 to 4	46 to 72	4.0	2.0
	Anidulafungin*	0.008 to 4	46 to 72	2.0	1.0

Abbreviations: MIC: Minimum Inhibitory Concentration, MEC: Minimum Effective Concentration, *MEC is recorded).

because of swimming in the lake water through the wound, as many studies have reported this in children and humans (Baumgartner et al., 2007; Chen et al., 2016; Signore et al., 2017). An increase in the overall incidence of *S. apiospermum* infection has been seen as a consequence of rampant use of steroids, broad spectrum-antibiotics, transplants, in cancer patients and through improved diagnostics (Sahi et al., 2007). Dissemination usually occurs during or immediately after increased immunosuppression, prolonged neutropenia and the use of antifungals which do not respond to the localized infection (Haynes et al., 2012; Chen et al., 2016). So, a localized infection caused by this pathogen should be addressed immediately. The diagnosis of this infection is also very difficult in veterinary profession as the animal owners do not provide complete history. As the organism is ubiquitous, the infection once developed, can become chronic without proper treatment and in severe/disseminated cases becomes fatal.

In the present case, a combination therapy was started and included Sporanox® capsules (Itraconazole) and Tablet Voritek® (Voriconazole) at 5 mg/kg body weight each while waiting for the AFST results. As pain management, Tablet Previcox® (Firocoxib) at 5 mg/kg body weight once daily for five days was prescribed. Over a two-week period, the skin lesions improved significantly; however, itching did not stop. No other side effects or adverse reactions were observed during the treatment regimen. Surgical debridement of the wound was not carried out as the owner denied the procedure. The combination therapy was continued for another four weeks. Complete wound healing was observed and no signs of itching or pain were observed in the dog during that period. The owner was advised to administer the antifungal treatment for another two months for complete removal of fungal elements and to avoid relapse, which is common in this fungal infection (Boyd et al., 2018; Kochenburger et al., 2019).

In this case, the majority of the antifungals exhibited high MIC/MEC values against *S. apiospermum* isolate (Table 1). The pathogen is commonly resistant against amphotericin-B, 5-flucytosine, caspofungin and fluconazole (Goldman et al., 2016). Voriconazole, which is the drug of choice in this infection, was found sensitive, which is in line with other studies (Chen et al., 2016; Signore et al., 2017). However, recurrences and development of resistance have been reported with voriconazole after a few weeks (Goldman et al., 2016; Boyd et al., 2018). Successful management and treatment of the infection require a combined use of antifungals and surgical debridement of infected tissues whenever possible (Signore et al., 2017, Boyd et al., 2018). Due to the lack of large studies in animals, little is known about the duration and efficacy of these antifungals. In the present case, combination therapy with itraconazole and voriconazole successfully treated the fungal infection; however, more in vivo studies are necessary to evaluate the efficacy of these antifungals.

CONCLUSION

As accurate and prompt diagnosis is essential, growth in culture media should be routinely followed by sequencing, which can provide results within a week. For effective and successful treatment of invasive mycosis, AFST should be carried out as a standard protocol (will take another three days) every two weeks since different fungal infection requires different antifungal treatment. This pathogen represents an important emerging fungal infection and there is a need to create awareness amongst pet-owners regarding Scedosporiosis and its consequences in dogs.

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