Antifungal sensitivity testing of Aspergillus species isolated from recurrent canine skin infection

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ABSTRACT

Aspergillus fumigatus, an opportunistic fungal pathogen, is responsible for skin infection in both humans and animals. The incidence of A. fumigatus dermatitis in canine as a primary pathogen is rarely reported. In this study, A. fumigatus and A. flavus were isolated from clinical samples of skin scrapings and infected hair from canines suffering from recurrent skin conditions. Sabouraud's dextrose agar (SDA) supplemented with chloramphenicol yielded typical velvety bluish-green colony of A. fumigatus and yellow-green cottony colony with sugary texture of A. flavus. The susceptibility of these isolates against anti-fungal drugs (ketoconazole and itraconazole) was evaluated by disk diffusion method. A. fumigatus was found resistant to ketoconazole but susceptible to itraconazole, whereas, A. flavus is susceptible to both ketoconazole and itraconazole. Antifungal susceptibility test for all fungal isolates should be routinely performed to prevent resistance against antifungal drugs used in veterinary dermatitis cases.

Key words: Antifungal sensitivity test, Aspergillus, Recurrent skin disease, SDA

INTRODUCTION

The genus *Aspergillus* has been classified into 8 distinct subgenera viz Aspergillus, Fumigati, Circumdati, Terrei, Nidulantes, Ornati, Warcupi, and Candidi (Peterson *et al*., 2008). More than 200 known species exist in the genus, but only a small percentage are associated with infections which includes pathogens such as, *Aspergillus fumigatus*, *A. flavus* and *A. niger* (Greub&Bille, 1998). *A. terreus* and *A. versicolor* are occasionally isolated from clinical specimens. The infections ranges from localized skin/nail/ocular infection to pulmonary disorder and invasive systemic infection collectively referred to as "*aspergillosis*," (Zhang *et al.*, 2012; Veraldi *et al.*, 2010; Ozer, *et al.*, 2009; Peeters & Clercx, 2007; Sharp & Matthews, 2006). Three major forms of aspergillosis such as nasal, bronchopulmonary, and disseminated infections occur in dogs caused by the primary pathogen *A. fumigatus*, followed closely by *A. flavus* and *A. niger* (Seyedmousavi *et al.*, 2015). However, incidence of *A. fumigatus* cutaneous dermatitis in canine species as a primary pathogen is rarely reported. The treatment of choice for these disease conditions is based on the use of antifungal drugs such as voriconazole, itraconazole, posaconazole, ketoconazole, thiabendazole and more recently, isavuconazole (Denardi, 2018). Nevertheless, *A. fumigatus* has been reported to develop intrinsic resistance to fluconazole and increased resistance to the azole antifungal drugs (Van der Linden *et al.*, 2011; Leonardelli *et al.*, 2016; Wiederhold, 2017). The widespread and increased use of azoles in the treatment of aspergillosis and their growing resistance to azoles group of drugs reiterate the importance of antifungal susceptibility studies to understand the resistance profile and improve treatment.

A.fumigatus and *A.flavus* were both isolated from clinical samples of two dogs with chronic recurrent skin problem which could contribute to contamination of their environment with pathogenic fungal spores, causing great public health concern. The aim of this paper is to evaluate both these isolates and their antifungal susceptibility by disk diffusion method against commonly used antifungal agents in veterinary practice.

MATERIALS AND METHODS

Sample collection:

Hair and skin scraping were collected aseptically from two canine patients (a spayed female retriever-mixed and an intact rescued female dog) with recurrent skin infection (Figs. 1 & 2). Direct impression smears from the skin of both patients were also collected for preliminary staining with Lactophenol cotton blue (LPCB) stain and 10% KOH solution to confirm the presence of fungal spores.

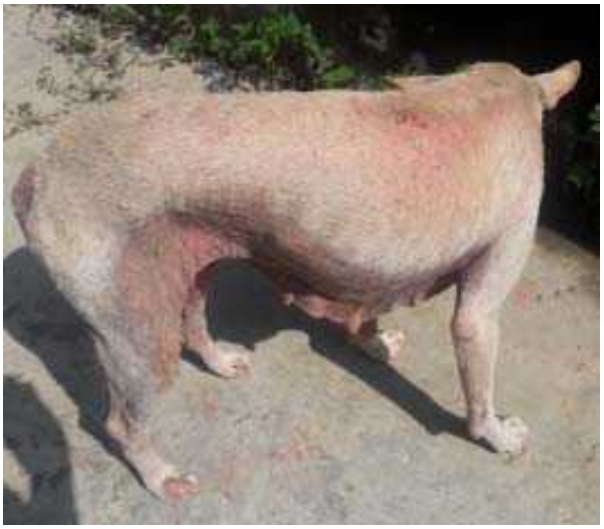

Fig. 1: Photograph of an intact female rescued mongrel weighing 27 kgs, with skin lesion on the ventral abdomen, caudal thighs, tail-head, neck extending to lateral chest and abdomen, face and over the dorsum of the body. (Patient no. 1)

Fig. 2: Photograph of a spayed female retriever-mixed weighing 28kgs with generalized papulonodular skin lesions on forelimbs and hindlimbs.(Patient no, 2)

Isolation of fungus:

Hair and skin scraping samples were inoculated on three separate plates of Sabouraud's Dextrose Agar (SDA) supplemented with chloramphenicol (0.05mg/l) and Dermatophyte test medium (DTM) to rule out dermatophytes infection. The inoculated plates were incubated at 25°C and 37°C, respectively, for 5 days with sufficient humidity. Fungal colonies were identified based on their colony characteristics and microscopic morphology of conidia and conidiophores as per standard protocols (Markey *et al.*, 2013).

Antifungal susceptibility test:

Five isolated colonies of *A. fumigatus* and *A. flavus* from clinical samples were further subcultured on SDA to obtain pure and adequate spores. The conidia were mixed in sterile saline solution and 0.05% Tween 20. Inoculum size was adjusted to a volume of 2 x 10^6 CFU/ml using a haemocytometer. Antifungal susceptibility against itraconazole and ketoconazole was determined using disk diffusion method on Mueller-Hinton Agar (MHA) containing 2% glucose and 0.5 µg/ml of methylene blue dye according to the guidelines of Clinical and Laboratory Standards Institute (CLSI)-M51-A (CLSI-M51-A).

RESULTS AND DISCUSSION

The initial staining of hair, skin scrapings and impression smears from the two patients indicated fungal spores of *Aspergillus* sp. Cutaneous aspergillosis is mainly caused by ubiquitous soil and water-dwelling saprophytes of the genus in immunocompromised patients. Although, skin infection caused by *A. fumigatus,* as a primary pathogen, has not been reported in dogs, *A. flavus* and *A. terreus* are commonly isolated. It has also been reported that outbreaks of Aspergillosis involving skin, oral mucosa or subcutaneous tissues are more often associated with A. flavusthan other species (Heinemann *et al.*, 2004; Hedayati *et al.*, 2007). In this study, two dogs showed similar cutaneous symptoms with formation of papules and nodules on the affected area of the body. Hedayati *et al*. (2007) also reported the presence of hemorrhagic bullae, ulcerations with central necrosis with or without eschar formation, pustules or subcutaneous abscesses in *A. flavus* in skin infections.

Isolation of fungus on DTM yield no growth even after 5 days of incubation, hence kept for further incubation at the required temperature and humidity. Inoculation of skin scrapings in SDA plates supplemented with chloramphenicol yielded mixed colony of *A. flavus* and *A. fumigatus* (Fig. 3a,3b& 4a). *A. fumigatus* is a fast grower; the colony size reached 3 cm within 5 days with a typical velvety bluish-green colony (Fig.3c). *A. flavus* showed velvety yellow color colony with a sugary texture, and reached 4 cm in size within 18 days of incubation (Fig. 3d). Microscopic examination of the morphology of conidia and conidiophores confirmed the typical the bluish-green color of *A. fumigatus* conidial heads and the clearly distinct yellowish-green conidial heads of *A. flavus*. The bluish-green color conidia were arranged in chains basipetally from greenish phialids borne directly on broadly clavate vesicles (20 to 30 mm in diameter) (Fig. 4b). *A. flavus,* on the other hand, showed

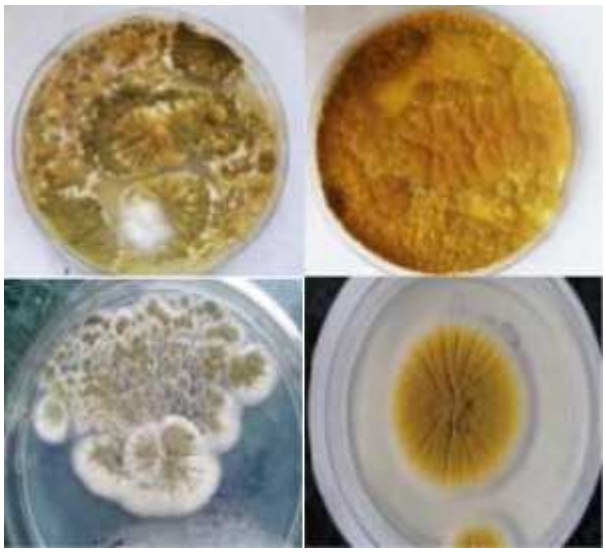

Fig. 3: Fungal colony consisting of *A. fumigatus and A. flavus* **(a)**mixed colonyisolated from skin scraping of patient no. 1 **(b)**mixed colony isolated from skin scraping of patient no. 2. **(c)**purecolony of *A. fumigatus* isolated from hair sample of patient no.1 **(d)** pure colony of *A. flavus* isolated from hair sample of patient no. 2

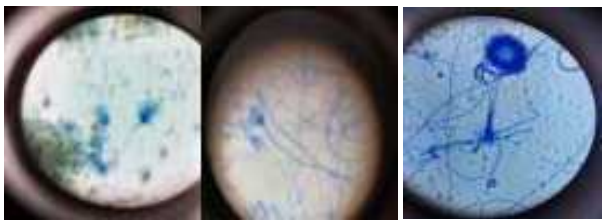

Fig. 4: Lacto-phenol cotton blue stain of colonies, 400X **(a)** Mixed *A. fumigatus* and *A. flavus* isolated from skin scrapingsof patient no. 1 and 2. **(b)** *A. fumigatus* isolated from hair sample of patient no. 1 **(c)** *A. flavus* isolated from hair sample of patient no. 2.

distinct yellowish-green radiating conidial heads arranged in chains from phialids which arised circumferentially from a globose vesicle (Fig. 4c). (Markey *et al.*, 2013).

Species differentiation is clinically important for selection of appropriate antifungal drugs (Lass-Flöri, 2014). Antifungal sensitivity test of *A. fumigatus* showed resistant to ketoconazole but susceptible to itraconazole. (Fig. 5a). Whereas, *A. flavus* was found susceptible to both ketoconazole and itraconazole, with the latter having a more significant zone of inhibition (Fig. 5b & Table 1). Azole groups were selected here as the drug of choice even though increased resistance to the drug has been reported. Laboratory testing for resistance of other antifungal group of drugs such as polyenes and echinocandins is difficult, and their mechanisms of resistance are largely unknown. Hence, further pre-clinical and clinical studies are needed to further improve the methods for *in vitro* susceptibility

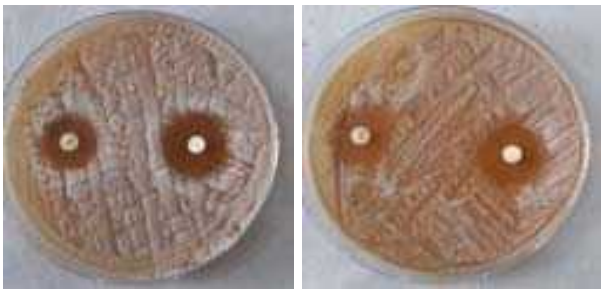

Fig. 5: Antifungal sensitivity test **(a)** *A.fumigatus* with resistance to ketoconazole. **(b)** *A. flavus* showed sensitivity to both ketoconazole and itraconazo

testing and to investigate the impact of elevated minimum inhibitory concentrations on antifungal drug efficacy.

Table 1: Antifungal sensitivity test by disk diffusion method (CLSI-M51-A)

Fungal Isolates	Ketoconazole (KT)	Itraconazole (IT)
A. fumigatus	R	S
A. flavus	S	S

R= resistant; S= sensitivity

CONCLUSIONS

Isolation of *Aspergillus* species from superficial clinical samples indicatedits pathogenicity to animals with an immune compromised system. There is also the possibility of pet dogs in spreading fungal spores within their immediate environment. Furthermore, antifungal susceptibility test for all fungal isolates should be routinely performed to evade an increase in fungal resistance to the limited antifungal drugs used in veterinary practice. For specific therapeutic dosage, minimum inhibition concentration (MIC) of each sensitive drug should be performed further.

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