DUAL MYCOTIC PULMONARY GRANULOMAS CAUSED BY *ALTERNARIA ALTERNATA* AND *ASPERGILLUS CANDIDUS* IN THE WILD EGYPTIAN MOLE RAT (*SPALAX LEUCODON* EGYPTIACUS)

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ABSTRACT

The population of the wild Egyptian lesser blind mole rat (*Spalax leucodon* Egyptiacus) is in decline, but the pathologies contributing to this are uncertain. Here we establish the cause of death in seven blind mole rats that died naturally in the wild. All the animals had large pulmonary lesions that on microscopic, microbiological, and ultrastructural analysis were shown to contain mixed infections with *Alternaria alternata* and *Aspergillus candidus*. Some of the lesions were circumscribed with fibroblastic proliferation and inflammatory response. The lungs had haemorrhage and chronic inflammatory response to the organisms, which is likely to have been the cause of death. This is the first report of some pathogenic organisms resulting in death of the blind mole rat.

Keywords: *Spalax leucodon; Lesser blind mole rat; Alternaria alternata; Aspergillus candidus.*

INTRODUTION

The Lesser blind Mole Rat "Spalax leucodon" (Harrison and Bates 1991) is a species of rodent in the Spalacidae family which is considered as subterranean rodent or burrowing mammal that find their food and spend most of their life underground (Hubalek *et al.,* 2005) and the only Egyptian mammal without apparent eyes (Richard Hoath, 2003). According to the International Union for Conservationof Nature and Natural Resources its Population is characterized by an acute restriction in its area of occupancy and becoming Critically Endangered or even Extinct in a very short period so it is categorized as Vulnerable (VU) D 2 in the IUCN Red List of Threatened species. According to the location of the animal we give it the name Egyptian lesser blind mole rat (Spalax leucodon egyptiacus). This study was conducted to determine the main causes of death in the Egyptian lesser blind mole rat" *S.l. egyptiacus*".

MATERIALS and METHODS

Seven wild Egyptian lesser blind mole rats (*Spalax leucodon Egyptiacus*) were found dead between March 2009 and March 2012 in the northern part of the Western Desert (Al Dabaa, Marsa Matroh Governorate) and were thought to have died from natural causes.

In order to establish the exact cause of death, each animal underwent thorough macroscopic and microscopic post-mortem examination. Affected lung tissues were removed, fixed in 10% buffered formalin, and then paraffin-wax embedded for light microscopic histologic examination of 3μm-thick sections mounted on slides and stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Ziehl-Neelsen (ZN) stains. Tissue samples were also prepared for transmission electron microscopy. Briefly, tissue samples were perfused with modified Karnovsky solution containing 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M (pH 7.3) 2.5% sodium cacodilate buffer, and fixed in the same solution for 12 hours at 4 °C. Specimens were post-fixed in 2% osmium tetroxide solution, rinsed in distilled water, and immersed in 2% tannic acid solution for 1 hour at room temperature (Murakami *et al.,* 1977). The tissues were then dehydrated in a graded ethanol series and propylene oxide before being embedded in Spurr resin. Semithin sections were prepared using a Porter Blum ultra microtome using glass knives and stained with toluidine blue solution for light microscopic analysis. Ultrathin sections were prepared using an ultra microtome (Ultra-Cut Reichert with diamond knife). The ultrathin sections were mounted on 200 and 300 mesh grids, counterstained with uranyl acetate and lead citrate, and examined using the Joel JSM1010 transmission electron microscope at 100 kV. For microbiological analysis, tissue samples were

smeared on the surface of Sabouraud dextrose agar in duplicate and incubated at both 25°C and 37°C. Growing colonies were subcultured at the edges of small pieces of agar on slides (microcultures) in order to study the microscopic structure of the isolated fungi.

RESULTS

On external examination, all the animals were emaciated and there was an absence of adipose tissue in the subcutaneous tissues and around the visceral organs. The most conspicuous macroscopic feature was the presence of discrete pulmonary lesions ranging from 0.25 -1 cm in diameter (Figure 1A), to yellowish-brown multifocal discrete but coalescing firm masses, ranging in size from 2 to 5 mm in diameter (Figure 1A). Generalized pulmonary oedema, emphysema (Figure 1B), and red hepatisation (Figure 1B) were seen in the background lung parenchyma.

Histological examination of the lungs revealed necrotizing granulomatous inflammation visible as necrotic-caseous material surrounded by, foamy macrophages, lymphocytes, and plasma cells (Figure 2A). There was severe congestion with marked alveolar destruction and haemorrhage in the background lung, and hemosiderin pigmentation as evidence of old haemorrhage within alveolar macrophages (Figure 2B). The wall of the larger lesions was comprised of a proliferation of large cells with slightly pleomorphic nuclei, vesicular chromatin, and grooved and folded nuclei, with occasional mitotic figures seen (Figure 2C). Semithin films stained with toluidine blue revealed the presence of large numbers of fungal organisms engulfed by macrophages, and transmission electron microscopy of the pulmonary tissue confirmed the presence of fungal hyphae associated with foamy macrophages (Figure 2D).

Two different colonies were isolated from the lesions of the animals. The first contained white, typically globose conidial heads producing globose, smooth, thin-walled conidia measuring 2.5–3.5 µm in diameter, typical of *Aspergillus candidus*. The second isolate showed features of *Alternaria alternata*, with fast growing, pale brown and smooth-walled organisms with a suede-like texture. Microscopically, branched acropetal chains (blastocatenate) of multi-celled conidia (dictyoconidia) extended sympodially from simple, sometimes branched, short or elongate conidiophores. The conidia were either obpyriform to ovoid, often with a short conical or cylindrical beak (Figure 3). Ziehl–Neelsen staining showed no evidence of mycobacteria and the other organs were normal.

The animals were therefore diagnosed with dual mycotic granulomas infected with *Aspergillus candidus* and *Alternaria alternata*. The granulomas elicited fibroblastic responses around the lesions, and the animals are likely to have died from respiratory failure.

Figure legends

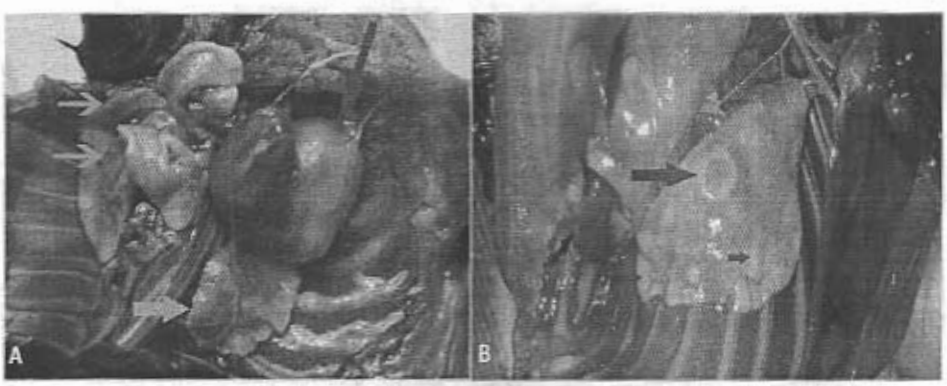

Figure 1: Macroscopic features. (A) Thoracic cavity of *Spalax leucodon* showing large, tumour-like lesions replacing the apical left lung lobe and large, firm brown lesions in the caudal and right lobes (yellow arrows). (B) Thoracic cavity containing brown, consolidating focal lesions in the centre of the left lung lobe, emphysematous change (red arrows), and red hepatisation of the lower right lobe (violet arrow).

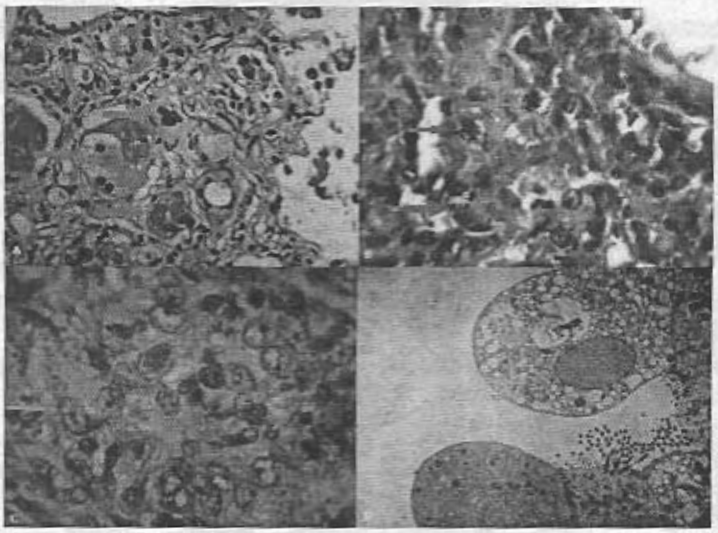

Figure 2: Microscopic and ultrastructural features. (A) Histological features of the lesions demonstrating fungal organisms within foamy macrophages (H&E x40). (B) Alveolar macrophages engulfing hemosiderin pigment (H&E x40). (C) Aspergilloma wall showing fibroblastic proliferation with slightly pleomorphic nuclei, vesicular chromatin, and grooved and folded nuclei (H&E x100). (D) TEM photomicrograph demonstrating fungal hyphae being engulfed by a foamy macrophage (X1000).

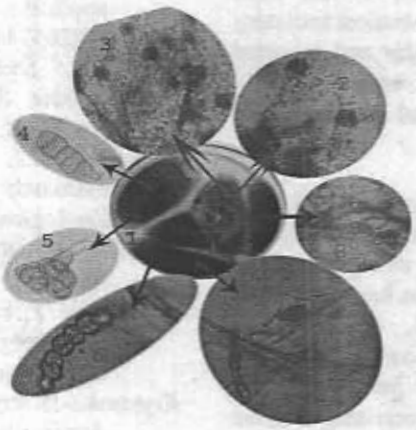

Figure 3: Colonies (1) and microscopic morphology of *Aspergillus candidus* (white) possessing white, globose conidial heads producing thin-walled conidia measuring 2.5–3.5 µm in diameter (2), and *Alternaria alternate* (black colonies) showing branched acropetal chains and multicelled, obclavate to obpyriform conidia with short conical beaks (3-8).

DISCUSSION

The population density of the Egyptian lesser blind mole rat (*Spalax leucodon Egyptiacus*) has dramatically decreased over the last few years (Tamam and omar 2009), and is categorised as vulnerable (VU; at high risk of extinction in the wild) in the IUCN Red List of threatened species (http://www.iucnredlist.org/). Agriculture and tourism have destroyed its natural habitat in the north coastal desert region of Egypt to the extent it no longer inhabits this region, although the species is thought to be present in small numbers in the south. Although destructive human activity is known to have affected species numbers, there are almost no studies investigating the pathogens that might contribute to species decline. This prompted us to undertake thorough post-mortem investigations on seven animals that died in the wild from seemingly natural causes unrelated to habitat destruction.

We have previously reported that the Egyptian lesser blind mole rat is frequently infested with the lynxacarus mite, which causes alopecia, gingivitis, and periodontitis in the animals (Tamam and omar

2009). Here we describe two more organisms that are pathogenic in *Spalax leucodon*, *Aspergillus candidus* and *Alternaria alternata*, which gave rise to severe and fatal pulmonary lesions. The inflammatory and fibrotic reaction occurring around the fungal lesions was consistent with a tissue reaction to pathogenic fungal infection, and the parenchymal lung changes secondary to severe infection.

Aspergillosis is a mycotic disease caused by a variety of species of the dimorphic fungus *Aspergillus*. Pulmonary aspergillosis is more usually caused by *Aspergillus fumigatus*, which is opportunistic in immunocompromised hosts, although rare cases of aspergilloma caused by *Aspergillus candidus* in man are described in the literature (Ribeiro S C. *et al.*, 2005). While *Aspergillus* is not usually a zoonotic or contagious disease, on exposure to an environmental source of *Aspergillus* spp., both birds and humans can develop an acute respiratory infection that has the potential to spread to other organs. Environmental exposure to *Aspergillus candidus* is a potential respiratory hazard for grain workers, with spores growing in large quantities in grain dust and contaminating air [4]. *A. candida* is not immunologically inert in mammals; it has been shown to possess strong immunostimulatory properties, inducing a specific cellular and humoral response in experimentally-exposed animals and in grain workers occupationally exposed to this fungus (Krysinska and Dutkiewicz, 2000).

Aspergillus candidus is pathogenic in pigs, and there is some evidence that *A. candidus* might be toxic to chickens and rats and that it can act as a pathogen to zoo animals and birds. The organisms have also been found on dead bees (Batra *et al.*, 1973). Taken together, these data suggest that *A. candidus* has the potential to be pathogenic under certain circumstances and it is therefore unsurprising that the organism was detected in the pulmonary lesions in the blind mole rat.

The terms alternariosis and alternariatoxicosis refer to disorders occurring in humans and animals caused by fungi belonging to this genus. *Alternaria alternata* is an air borne disease that can give rise to diseases in plants, animals and humans, and the organism is not infrequently cultured in healthy animals (Andersen *et al.*, 2001). *Alternaria alternata* is one of the causative agents of phaeohyphomycosis, and cases of onychomycosis, sinusitis, ulcerated cutaneous infections, and keratitis, as well as visceral infections and osteomyelitis, have been reported to occur due to infection with *Alternaria* (Anaissie *et al.*, 1989; Manning *et al.*, 1991). It is among the causative agents of otitis media in agricultural field workers (Wadhwani and Srivastava, 1984). Although *Alternaria* are ubiquitous organisms, they certainly are potential pathogens and therefore their co-isolation, along with *Aspergillus candidus* in this case, is consistent with being causative of the pulmonary lesions observed in these animals.

In conclusion, here we describe for the first time severe fatal lung lesions occurring in the blind mole rat, caused by *Aspergillus candidus* and *Alternaria alternata*. These observations point to the presence of some causes for population decline, and further investigation of other causes is warranted.

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الاورام الرئوية لحيوان ابو عماية نتيجة للاصابة المزدوجة بفطر الاسبيرجيلس كانديداس والالتيرناريا التيرناريا

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حيوان ابو عماية من الثدييات المعرضة للتناقص المستمر في ظل غياب الدراسات البيئية والباثولوجية حول هذا الحيوان وأسباب تناقصه. تناولت هذه الدراسة أسباب نفوق سبعة حيوانات في بيئتها الطبيعية حيث خضعت هذه الحيوانات إلى العديد من الفحوصات الإكلينيكية ابتداء من التشريح المرضى ثم تم الاستعانة في فحص العينات على الميكروسكوب الضوئي والالكتروني .اظهر الدراسات إصابة جميع الحيوانات بإصابات متفاوتة بالرئتين ابتداء من احتقان النسيج الرئوي مع ارتشاحات دموية ووجود بعض التورمات بالرئتين. تم عزل كل من فطر الاسبيرجيلس كانديدس والالتيرناريا التيرناريا كإصابة مزدوجة في كل الحالات. بعض الإصابات الرئوية تظهر مجهريا كأشكال دائرية تحيطها الخلايا الليفية والخلايا المصاحبة للالتهابات المزمنة مع زيادة حدة الانزفة في الأنسجة المحيطة بالأورام الفطرية وهذا يوضح أسباب نفوق هذه الحيوانات. هذه الدراسة تعد الأولى التي تلقى الضوء حول الأمراض التي تصيب هذا الحيوان وأسباب نفوقه.