Attempted molecular detection of the thermally dimorphic human fungal pathogen Emergomyces africanus in terrestrial small mammals in South Africa

Short title: Emergomyces africanus in small mammals

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Abstract

The ecological niche of *Emergomyces africanus (*formerly *Emmonsia species)*, a dimorphic fungus that causes an AIDS-related mycosis in South Africa, is unknown. We hypothesized that natural infection with *E. africanus* occurs in wild small mammals. Using molecular detection with primers specific for *E. africanus*, we examined 1402 DNA samples from 26 species of mole-rats, rodents and insectivores trapped in South Africa that included 1324 lung, 37 kidney, and 41 liver specimens. DNA of *E. africanus* was not detected in any animals. We conclude that natural infection of wild small mammals in South Africa with *E. africanus* has not been proven.

Emergomyces africanus is a recently described thermally dimorphic fungal pathogen (formerly an *Emmonsia* species)[1] responsible for emergomycosis (formerly disseminated emmonsiosis), an AIDS-related systemic mycosis with a high case-fatality rate in southern Africa[2,3].

The geographic range of *E. africanus* is currently being defined, but to date human cases have been reported from six of nine South African provinces (Western Cape, Eastern Cape, Gauteng, Free State, Mpumalanga[3], KwaZulu-Natal[4]) and from the Kingdom of Lesotho[2].

The ecological niche of *E*. *africanus* in southern Africa is unknown, although soil is presumed to be the reservoir [5]. The presence of an alternative mammalian host has been proposed as a possible explanation for the restricted geographic range of *E. africanus* [6]. For some other medically important dimorphic fungi with restricted geographic ranges, particular mammals frequently are naturally infected, and may serve as sentinel species which help to map out the geographic range[7]. For instance, *Talaromyces marneffei* (formerly *Penicillium marneffei*)[8], the cause of talaromycosis, and *Paracoccidioides* spp*.,* the causes of paracoccidioidomycosis, are restricted to southeast Asia and South America, respectively. Both are difficult to detect in the environment, but relatively easy to detect via microscopy, culture and molecular testing of samples from bamboo rats (*Rhizomys* species and *Cannomys badius*) and armadillos (*Dasypus novemcinctus* and *Cabassous centralis*), respectively[7].

In this study, we used molecular detection to screen (predominantly) the lungs of mole-rats, rodents and insectivores from various regions in South Africa to elucidate the ecological niche of *E. africanus*.

Samples originated from small mammals previously trapped for unrelated studies. Mole-rats were trapped across natural, semi-natural, and urban sites in the Northern and Western Cape provinces. The other rodents and insectivores were sampled from natural and semi-natural localities in the Western Cape, Eastern Cape, Free-State, KwaZulu-Natal, Northern Cape, North West and Gauteng Province of South Africa. Animal trapping, handling and euthanasia were performed with approval from the research ethics committees of Stellenbosch University and University of Pretoria (see Supplemental Digital Content S1 for animal ethics approval and permit details).

Nucleic acids had been extracted previously[9] from homogenised pieces of lung, liver, and kidney samples using the RNeasy® Mini kit (Qiagen, Germany) following the spin protocol on the QIAcube® automated extraction platform. Tissue samples were homogenized in lysis buffer (containing β-mercaptoethanol) with either the TissueLyser LT homogeniser (Qiagen, Germany) or by repeatedly passing the tissue sample, finely chopped, through a 2-ml syringe using a 22-gauge needle. Samples were then centrifuged and the cell-free supernatant was used as starting material as per the manufacturer's protocol. To extract total nucleic acids, the DNase treatment step in the protocol was omitted.

A conventional PCR protocol using TrueStart Hot Start DNA *Taq* polymerase (Thermo Fisher Scientific, USA) was used to screen samples for the presence of *E. africanus*. Primers, NEF (forward) 5' CGGGGACACCGTAGAAAACACT 3' and NER (reverse) 5'GAGATCCGTTGTTGAAAAGTTTTAACT 3', were designed targeting an 80 bp fragment of the internal transcribed spacer (ITS) region of ribosomal RNA specific for *E. africanus*. Briefly, 20 µl reactions consisted of nuclease-free water, PCR reaction buffer, $MgCl_2$ at a final concentration of 1.25 mM, primers at a final concentration of 0.4 µM each, dNTPs at 250 µM final concentration each, 1.25 U *Taq* polymerase with 2 µl template DNA. Thermocycling conditions consisted of 5 minutes at 95°C, followed by 35 cycles of 94 °C for 30 seconds, 55 °C for 1 minute, and 72 °C for 2 minutes, with a final extension time of 7 minutes at 72 °C.

PCR sensitivity was previously assessed on extracted DNA from an *E. africanus* cultured isolate. The extracted DNA concentration was determined spectrophotometrically and following a series of dilutions, the lowest concentration detected by PCR was 20 pg/ml.

Alongside each run, positive controls were used. For testing of mole-rats, the positive control was a clinical strain of *E. africanus* (provided by NICD, GenBank accession: JX398296) cultured on malt extract agar medium at 25 °C. Extracted DNA from rodent lung samples spiked with either the mould or yeast phase of *E. africanus* prior to homogenization and extraction was used as positive control material. For the mould phase, clinical isolates were provided by NICD (GenBank: JX398291 and JX398293)

and for the yeast phase, a clinical isolate was provided by the National Health Laboratory Services at Tygerberg General Hospital.

We tested 1402 animals, including lung, kidney, and liver samples from 1324, 37, and 41 animals, respectively. The animals comprised 26 species (Table 1). For all samples screened, only positive controls produced the amplicon size of interest. All other samples were negative by PCR.

In summary, we screened a wide range of small mammals throughout South Africa for clues to the ecological niche of *E. africanus*. We failed to detect *E. africanus* in the lungs and other tissues of 26 small mammal species from areas where human disease caused by *E. africanus* infection is endemic.

There are several possible limitations of this study. Firstly, the possibility exists that our protocol for processing tissue for fungal DNA extraction was flawed, or the sensitivity of the PCR assay was insufficient, resulting in falsely negative results. Ideally, a positive control would include the tissues of an animal exposed to aerosolized *E. africanus* conidia. This was not possible for logistical, biosafety and bioethical reasons. Instead, we tried to simulate natural infection by spiking lung tissue with *E. africanus* moulds and yeasts. These spiked samples were successfully amplified although it is noted that the amount of DNA in spiked tissue is likely much higher than in natural infection. We cannot exclude the possibility that natural infection could fall below the level of detection for our assay. Despite not detecting *E. africanus* in any of these samples, a range of

Table 1. Geographic and species distribution of mole-rat, rodent, and insectivore samples screened for Emergomyces africanus infection.

Species	Common name	Province							
		WC	NC	FS	G	EC	KZN	NW	Total
Family Batdyergidae									
Bathyergus suillus	Cape dune mole-rat	86							**86**
Cryptomys hottentotus hottentotus	common mole-rat	6	39						**45**
Fukomys damarensis	Damaraland mole-rat		50						**50**
Georychus capensis	Cape mole-rat	53							**53**
Family Muridae									
Aethomys sp.	rock rats, bush rats							8	**8**
Aethomys chrysophilus	red rock rat							4	**4**
Gerbillurus paeba	hairy-footed gerbil		16						**16**
Lemniscomys rosalia	single-striped grass mouse						1	13	**14**
Mastomys coucha	Southern mulitmammate mouse		1						**1**
Mastomys natalensis	Natal multimammate mouse					7	10		**17**
Mastomys sp.	multimammate mice, Natal-rats				24			3	**27**
Micaelamys namaquensis	Namaqua rock rat	39		26		8	14		**87**
Mus inputs	desert pygmy mouse							3	**3**
Mus minutoides	African pygmy mouse	3					1	7	**11**
Mus musculus	house mouse	1	6			6			**13**
Otomys irroratus	Southern African vela rat	15				30	12		**57**
Otomys sp.	African Karoo rats, vlei rats				1		2		**3**
Myotomys unisulcatus	bush vlei rat	21	4						**25**
Rattus norvegicus	brown rat, common rat					3			**3**
Rattus rats	black rat, house rat	13				2	1		**16**
Rhabdomys pumilio	four-striped grass mouse	475	100	3	53	63	51	18	**763**
Tat era brantsii	Highveld gerbil							17	**17**
Family Nesomyidae									
Steatomys pratensis	fat mouse							10	**10**
Family Macroscelididae	elephant shrews	10	2	2					**14**
Family Soricidae	shrews	15	1			7	33	3	**59**
Total		**737**	**219**	**31**	**78**	**126**	**125**	**86**	**1402**

EC, Eastern Cape province; FS, Free State province; G, Gauteng province; KZN, KwaZulu-Natal province; NC, Northern Cape province; NW, North West province; WC, Western Cape province.

fungal species were detected in rodent (Muridae), shrew (Soricidae) and mole-rat (Bathyergidae and Spalacidae) samples using the pan-fungal ITS4 and ITS5 primers, attesting to adequate extraction of fungal DNA (Supplemental Digital Content S2).

Secondly, the finding of only negative results begs the question about sample integrity. To assess the integrity of stored extracted DNA sample, the cytochrome *b* gene was successful amplified across a randomly selected subset of samples (Supplemental Digital Content S3). Furthermore, viral RNA, easily destroyed with poor sample care and storage, has been detected in these same samples during ongoing studies (unpublished), suggesting that sample integrity has been maintained.

Thirdly, animal tissues were not evaluated with non-molecular detection techniques such as histopathological examination and culture. In other studies, the sensitivity of molecular detection approached or exceeded that of culture-based methods (for example, in ecological[10,11], experimental[12], and clinical studies of *Paracoccidioides*). Lastly, in most cases only lungs were tested. Given that inhalation of conidia is thought to be the primary route of inoculation with *E. africanus*[5]*,* we expected lung tissue to have the highest yield.

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Conflicts of Interest: None.

References

1. Dukik K, Muñoz JF, Jiang Y, et al. Novel taxa of thermally dimorphic systemic pathogens in the Ajellomycetaceae (Onygenales). *Mycoses*. 2017. doi:10.1111/myc.12601.
2. Kenyon C, Bonorchis K, Corcoran C, et al. A dimorphic fungus causing disseminated infection in South Africa. *N Engl J Med*. 2013;369(15):1416-1424. doi:10.1056/NEJMoa1215460.
3. Schwartz IS, Govender NP, Corcoran C, et al. Clinical characteristics, diagnosis, management and outcomes of disseminated emmonsiosis: a retrospective case series. *Clin Infect Dis*. 2015;61(6):1004-1012. doi:10.1093/cid/civ439.
4. Maphanga TG, Britz E, Zulu TG, et al. In vitro antifungal susceptibility of the yeast- and mould-phases of the dimorphic fungal pathogen, Emergomyces africanus (formerly Emmonsia species), from HIV-infected South African patients. *J Clin Microbiol*. 2017. doi:10.1128/JCM.02524-16.
5. Schwartz IS, Kenyon C, Feng P, et al. 50 Years of Emmonsia Disease in Humans: The Dramatic Emergence of a Cluster of Novel Fungal Pathogens. *PLoS Pathog*. 2015;11(11):e1005198. doi:10.1371/journal.ppat.1005198.
6. Latgé J-P. Oh, to be new. *N Engl J Med*. 2013;369(15):1464-1466. doi:10.1056/NEJMe1309132.
7. Restrepo A, Baumgardner DJ, Bagagli E, et al. Clues to the presence of pathogenic fungi in certain environments. *Med Mycol*. 2000;38 Suppl 1:67-77.
8. Samson RA, Yilmaz N, Houbraken J, et al. Phylogeny and nomenclature of the genus Talaromyces and taxa accommodated in Penicillium subgenus

Biverticillium. *Stud Mycol*. 2011;70(1):159-183. doi:10.3114/sim.2011.70.04.

9. Witkowski PT, Klempa B, Ithete NL, et al. Hantaviruses in Africa. *Virus Res*. 2014;187:34-42. doi:10.1016/j.virusres.2013.12.039.

10. Sbeghen MR, Zanata TB, Macagnan R, et al. Paracoccidioides brasiliensis Infection in Small Wild Mammals. *Mycopathologia*. 2015;180(5-6):435-440. doi:10.1007/s11046-015-9928-8.

11. Arantes TD, Theodoro RC, Teixeira M de M, Bosco S de MG, Bagagli E. Environmental Mapping of Paracoccidioides spp. in Brazil Reveals New Clues into Genetic Diversity, Biogeography and Wild Host Association. *PLoS Negl Trop Dis*. 2016;10(4):e0004606. doi:10.1371/journal.pntd.0004606.

12. Bialek R, Ibricevic A, Aepinus C, et al. Detection of Paracoccidioides brasiliensis in tissue samples by a nested PCR assay. *J Clin Microbiol*. 2000;38(8):2940-2942.