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Emergence of *Cryptococcus* spp. in Donkeys in Egypt: A Potential Public Health Concern

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ABSTRACT

Cryptococcus has gained medical importance over the last decade, as it is an emerging pathogen among immunocompetent individuals. There are no epidemiological data on the prevalence of this fungus in donkeys. The current research was conducted to investigate the possible role of the Egyptian donkeys in the epidemiology of such pathogen. Bacteriological analysis of nasal swabs of 52 diseased and healthy donkeys at different localities in Egypt revealed that the overall occurrence of *Cryptococcus* spp. was 11.5%. The highest proportion was in El-Fayoum Governorate (25). Phenotypic identification of *Cryptococcus* indicated that 13.2 % and 7.1% among healthy and diseased donkeys were positive for this pathogen, respectively.The study of the potential risk factors associated with *Cryptococcus* colonization in the donkeys did not show any statistically significant differences. Molecular serotyping of 6 identified *Cryptococcus* spp. evidenced *C. gattii* in the nasal passages of 4 healthy donkeys (7.7%); while the other 2 *C. neoformans* serotype A (3.8%) isolates identified in healthy and diseased donkeys. Four *C. gattii* and *C. neoformans* isolates demonstrated higher laccase (*LAC1*) genes among the identified virulence factors. While capsular associated protein (*CAP59*) gene identified alone or associated with *LAC1* gene in the other 2 *C. gattii* isolates. This study underlines a potential association of those fungi with human disease in Egypt. In order to strengthen existing therapeutic and control approaches, further analyses of the main risk factors and the other virulence of these pathogens should be further considered.

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Authors' Contribution
RM and SN collected and prepared the samples, applied bacteriological analysis and PCR assay. DH helped in laboratory work, reviewing and editing. MAS supervised the study, data curation and wrote the manuscript.

Key words
Donkeys, *Cryptococcus neoformans*, *Cryptococcus gattii*, Nasal swabs, Potential risk factors, Virulence factors, *Laccase* gene, Capsular associated protein gene.

INTRODUCTION

Recently fungal pathogens, *Cryptococcus* spp. have increasingly been recognized as a major threat to the populations' health worldwide. There are at least 37 distinct *Cryptococcus* spp. of which *Cryptococcus neoformans* and *Cryptococcus gattii* are human pathogens (Kwon-Chung *et al*., 2017). *C. neoformans* taxonomic classification illustrated *C. neoformans* and *C. deneoformans* as *C. neoformans* serotype A has three genotypes: VNI, VNII, and VNB and *C. deneoformans* serotype D has a genotype VNIV. In the case of *C. gattii,* there are five cryptic species with serotypes B, C and genotype from VGI to IV (Hagen *et al*., 2015). While all of the serotypes can vary in their topographical distribution, they can all cause disease in humans and animals. Annually, approximately 625,000 deaths are reported for one million cases of cryptococcal meningitis among people with HIV/AIDS due to infection with those species (Centers for Disease Control and Prevention, CDC, Atlanta, USA, http://www.cdc.gov/). *C. gattii* appears to have a greater propensity to infect immune-competent humans (Rozenbaum and Gonçalves, 1994; Speed and Dunt, 1995). The infection is transmitted from environment by inhalation of spores or dehydrated yeast cells that can enter the pulmonary alveoli and then spread through the bloodstream causing respiratory disorders such as pneumonia, soft tissue disease, and most frequently meningoencephalitis (Kwon-Chung *et al*., 2014).

As a result of the environmental changes, the number of fungal diseases in animals and plants has increased (Fisher *et al*., 2012). *C. neoformans* have not only been isolated from avian excreta but also from soil and house dust (Swinne *et al*., 1986; Irokanulo *et al*., 1997; Litvintseva *et al*., 2011) as well as exotic, migratory birds, domestic and wild animals may be carriers or susceptible hosts for this species (Casadevall and Perfect, 1998). Additionally, *C. gattii* species complex can colonize the plethora of tree species (Vélez and Escandón, 2017).

In Egypt, Saleh (2005) isolated *C. neoformans* from vaginal swabs examined from different animal species. The same pathogen (*C. neoformans*) was also isolated from throat and vaginal swabs from women rearing pigeons (Saleh *et al*., 2011). Environmental surveys conducted in eight African countries including Egypt showed that these pathogens represented 1% of the total recorded environmental isolates (Cogliati, 2013).

The last statistics from Food and Agriculture Organization (FAO) estimates that about 3.3 million donkeys (*Equus asinus*) live in Egypt. A vast majority of these donkeys are daily working animals and they form the

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country's second largest population of livestock after goats in the region. Similar to other mammals, as reported by Fisher *et al*. (2012), donkeys and horses may be affected by several fungal diseases which pose a serious threat to them. In horses, cryptococcosis is primarily associated with respiratory tract disorders, central nervous system (CNS), and premature birth. Disseminated cryptococcosis is documented in horses (Zoppa *et al*., 2008), whereas cutaneous cryptococcosis in donkeys was reported (Khodakaram-Tafti and Dehghani, 2006).

The health of these working animals is closely linked to the health of the human population, from the one health principle. Therefore, there is an urgent need to investigate the role of donkeys in Egypt's cryptococcal epidemiological process. Since no epidemiological data are available in Egypt among cryptococcal infection in donkeys, the current research was conducted to investigate the occurrence of *Cryptococcus* species among healthy and diseased donkeys and to determine their serotypes and virulence factors.

MATERIALS AND METHODS

Samples collection and preparation

Nasal swabs were collected from donkeys raised in different localities in Cairo, Giza, and El-Fayoum Governorates. Donkeys included were 38 apparently healthy and 14 diseased suffering from wounds, mobility disorder, stomatitis, nasal discharge, ocular discharge or abscess. The swab samples were inoculated into sterile Sabouraud dextrose broth (Oxoid) supplemented with chloramphenicol (0.1g/L) (HiMedia), then were transported to the laboratory in ice box. Data from each individual animal were collected including age, sex and underlying health issues.

Protocol of samples collection was carried out in compliance with the recommendations of the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine, Cairo University, Egypt (VetCU20022020123).

Isolation and phenotypic identification of Cryptococcus *spp.*

According to Horta *et al*. (2002), the inoculated swab samples were incubated at 37°C for 24 h. Then the prepared sample supernatant was streaked onto plates of SDA with chloramphenicol, and was incubated for 48-72 h at 37°C. The colonies with a mucoid like appearance (Supplementary Fig. S1) were selected and were identified by microscopic morphology of yeast cells.

For identification of *Cryptococcus* isolates based on melanin synthesis, a loop from the original broth were streaked onto Tobacoo agar media (TAM) plates and incubated for 3-5 days at 37°C (Tendolkar *et al*., 2003; Refai *et al*., 2005). Biochemical identification of the colonies was done using RapID yeast plus system (RYP) (Remel, USA) (Smith *et al*., 1999; Soltani *et al*., 2013).

Molecular identification

Genomic DNA were extracted from the pure *Cryptococcus* isolates using boiling method according to Mohammadi *et al*. (2017).

Multiplex PCR was carried out using specific oligonucleotide primers (Table I) to detect *C. neoformans* serotype A and *C. gattii* serotype B. The PCR reactions were performed in a total volume of 25µl, containing 3µl of template DNA from each isolate, 12.5 µl of Master Mix (takara, Japan), 0.5µl of each primer (Metabion, Germany) and 7.5µl of PCR grade water.The PCR reaction mixtures were amplified using thermal profile conditions (Table II). The PCR amplicons were electrophoresed on agarose gel (1.5 %) at 100 V for 60 min and visualized under ultraviolet light.

Table I.- Sequence of oligonucleotide primers for molecular serotyping of *C. neoformans* and *C. gattii* and identification of the virulence genes in the isolates.

Target genes	Primer sequence (5'- 3')	Amplicon size (bp)	References
C. neoformans, CNa-70S *CNa-70A*	ATTGCGTCCACCAAGGAGCTC ATTGCGTCCATGTTACG TGGC	695	Aoki *et al*. (1999); Lusia-Leal *et al*. (2008)
C. gattii, CNb-49S *CNb-49A*	ATTGCGTCCAAGGTGTTGTTG ATTGCGTCCATCCA ACCGTTATC	448	
Laccase gene, *LAC1*	AACATGTTCCCTGGGCCTGTG ATGAGAATTGAATCGCCTTGT	469	Fraser *et al*. (2005); Meyer *et al*. (2009)
Capsular associated protein, *CAP59*	CTCTACGTCGAGCAAGTCAAG CCGCTGCACAAGTGATACCC	559	
Phospholipase, *PLB1*	CTTCAGGCGGAGAGAGGTTT GATTTGGCGTTGGTTTCAGT	532	Litvintseva *et al*. (2006); Meyer *et al*. (2009)

Table II.- PCR amplification thermal conditions of *C. neoformans* and *C. gattii* serotypes and virulence genes of the isolates.

Gene	Initial denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
CNa-70S *CNa-70A* *CNb-49S* *CNb-49A*	94°C 8 min	94°C 1 min	65°C 1 min	72°C 2 min	35	72°C 8 min.
LAC1	94°C 3 min.	94°C 30 sec.	58°C 30 sec.	72°C 1 min.	30	72°C 5 min.
CAP59	94°C 3 min	94°C 30 sec	56°C 30 sec.	72°C 1 min	35	72°C 5 min.
PLB1	94°C 3 min.	94°C 45 sec.	61°C 45 sec.	72°C 1 min	30	72°C 5 min

Molecular detection of the virulence genes

The extracted DNA from *Cryptococcus* spp. isolates were also examined for the presence of the virulence genes; laccase gene (*LAC1*), capsular associated protein (*CAP59*), and Phospholipase *PLB1*.

Uniplex PCR was performed using specific oligonucleotides primers shown in Table I. The PCR reaction mixtures of 25 µl total volume contain 12.5 µl of Master Mix (takara, Japan), 0.5µl of each primer (Metabion, Germany), 8.5 µl water and 3 µl template DNA from each isolate. Negative control was included which contains all the components of the PCR mixture, but with water instead of the template DNA. The PCR reaction mixtures were amplified using thermal profile conditions (Table II).

Statistical analysis

Data were collected, tabulated and statistically analysed with PASW, version 18.0, Software (SPSS Inc., Chicago, IL, USA). Fisher's Exact test and Fisher-Freeman-Halton Exact test (Freeman and Halton, 1951) (it is the Fisher's Exact test for contingency tables greater than 2x2) were used. Statistically significant *P*-value is less than 0.05.

RESULTS

Table III shows occurrence of *Cryptococcus* spp. in healthy (13.2%) and diseased (7.1%) donkeys. The highest percentage of occurrence of *Cryptococcus* spp. was in donkeys >10 year of age (14.3), while in both males (11.4%) and females (11.8%) the pathogen was almost identical (Table IV).

Molecular serotyping of 6 identified *Cryptococcus* spp. evidenced that *C. gattii* B was isolated from the nasal passages of four donkeys (7.7%), it was recovered from healthy examined donkeys, while the other 2 *C. neoformans* A isolates (3.8%) were identified in healthy and diseased donkeys (Table III). Clinical condition was recorded in only one 12 year -old male donkey (16.7%) with stomatitis among the 6 positive donkeys.

Table III.- Occurrence of *Cryptococcus* spp. among healthy and diseased donkeys.

Underlying health condition	No. of samples	Positive samples		
		C. neoformans	*C. gattii*	Total
Healthy	38	1	4	5
Diseased	14	1	0	1
Total	52	2	4	6

Table IV.- Occurrence of *Cryptococcus* spp. according to the age and gender of the examined donkeys.

Predisposing factors		No. of samples	No. of positive samples
Age (year)	1-5	33	4
	6-10	12	1
	>10	7	1
Total		52	6
Gender	Male	35	4
	Female	17	2
Total		52	6

Bacteriological examination of 52 nasal swabs collected from diseased and healthy donkeys at different localities in Egypt, evidenced that the overall occurrence

of *Cryptococcus* spp. was 11.5%. The highest percentage was reported in El-Fayoum Governorate (25%) followed by Cairo Governorate (10%). The lowest percentage of *Cryptococcus* spp. was recorded in Giza Governorate (8.8%) as shown in Table V. The statistical analysis showed that there is no significant difference ($P = 0.363$, Fisher's exact test) between the examined localities.

Table VI shows that laccase gene was the most frequently detected gene in 4 isolates of *C. gattii* (B) and *C. neoformans* (A). While capsular associated protein gene was found in the other two isolates of C. gattii (B) alone or associated with *LAC1*. The Phospholipase gene was however not identified in any species.

Table V.- Occurrence of *Cryptococcus* spp. in donkeys originated from different localities.

Location	No. of samples	No. of positive samples	% of positive samples
Cairo	10	1	10.0
Giza	34	3	8.8
El-Fayoum	8	2	25.0
Total	52	6	11.5

Table VI.- The virulence factors identified among *C. neoformans* and *C. gattii* isolates.

Serotypes	Virulence pattern			
	LAC1	***CAP59***	***LAC1 & CAP59***	***PLB1***
C. gattii (B), n=4	2	1	1	0
C. neoformans (A), n=2	2	0	0	0

DISCUSSION

The number of fungal and fungal-like diseases of plants and animals in both natural and controlled systems has increased over the last two decades, most likely as a result of the environmental changes (Fisher *et al*., 2012). As well as, the number of debilitated individuals is progressively increasing.

A significant number of literature focuses on individual clinical cases, whereas less is known about the disease epidemiology in horses (Duncan *et al*., 2011). To our knowledge, the role of donkeys has not been specifically investigated in this pathogen's epidemiology. In order to study the epidemiology of cryptococcosis, a diagnostic method is required to detect the presence of *Cryptococcus* spp. in serum, tissue samples, and nasal-swab samples (Krockenberger *et al*., 2003; Raso *et al*., 2004; Duncan *et al*., 2005, 2006a, b). In the current study, the overall recorded percentage of *Cryptococcus* spp. (11.5%) detected in nasal passages of the examined donkeys was nearly similar to those estimated by Danesi *et al*. (2014) who examined 766 cats nasal swabs and recovered *Cryptococcus* spp. from 95 (12.6%).

Our findings showed that apparently healthy donkeys are asymptomatic carriers of *Cryptococcus* spp. as, the highest occurrence of *Cryptococcus* spp. was detected in nasal passages of healthy examined donkeys, it indicates that the organism's environmental load in the studied area is significantly greater. In this context, Connolly *et al*. (1999) and Malik *et al*. (1997) reported that *Cryptococcus* environmental exposure and asymptomatic colonization of the respiratory tract much more common than clinical disease.

C. neoformans and *C. gattii* are commonly regarded as pathogenic species of the genus *Cryptococcus*. Molecular serotyping of the detected *Cryptococcus* spp. isolates in the current study revealed that *C. gattii* (B) was frequently detected among apparently healthy examined donkeys in relation to *C. neoformans* (A). Host factors that restrict the fungus to the respiratory tract without any symptoms may be attributed to incomplete elimination of *Cryptococcus* cells by alveolar macrophages that involved in host response against infection (Lin and Heitman, 2006).

The *C. gattii* fungal pathogen can infect hosts with and without an apparent immune defect. Duncan *et al*. (2005) recorded that asymptomatic carriage of *C. gattii* has been recognized in companion animal species of British Columbia, Canada, with most of the reported individuals remaining asymptomatic.

Lately, *C. gattii* came to public consciousness due to the outbreak of devastating disease in immunocompetent people. The first case of *C. neoformans* var. *gattii* serotype (B) from Egypt was detected in an HIV patient (Mansour *et al*., 2006). This serotype has also been identified as a potential main agent of granulomatous rhinitis in horses (Cruz *et al*., 2017). Species identification was necessary because *C. gattii* infections are increasingly considered alarming as it becomes more difficult to handle this fungus, because it is not susceptible to the most widely used antifungal agents (Trilles *et al*., 2004), as well as this pathogen infects the immunocompetent hosts, particularly children.

Age, sex, and health conditions of the individual animals have no statistically significant impact on *Cryptococcus* spp. nasal colonization as shown in the present study. This could be arguing for the presence of other risk factors such as the environment. Determining such possible factors can help animal-owners and veterinarians mitigate the risk of *Cryptococcus* spp.

infection.

The highest recorded occurrence of *Cryptococcus* spp. isolates in donkeys from El-Fayoum Governorate may reflect the environmental presence of Cryptococci which is presumably greater around the examined donkeys and this may be due to the presence of pigeons in the examined area. Chowdhary *et al*. (2012) and Datta *et al*. (2009) have confirmed this; they declared that *Cryptococcus* species are associated with environmental niches rich in avian guanos, particularly pigeon excreta (*C. neoformans*) and decaying vegetation.

Pathogenicity of a microbe relies on the existence of virulence factors that function to induce illness in unison. Since these factors are involved not only in pathogenesis but also in commensalism, some of the virulence genes have been molecularly identified among the isolates.

It is intriguing that all *C. neoformans* and *C. gattii* isolates in this study shared the same virulence factor, as they have laccase gene, this finding was confirmed by Ellerbroek *et al*. (2004) who declared *C. neoformans* and *C. gattii* have several common virulence factors.

The cryptococcal laccase determinant is a well-characterized virulence factor, producing a melanin cell wall coat that defends the cell against environmental factors; host attacks and antimycotic therapy less effectively cleanse it.

The absence of *CAP59* gene in the most researched isolates in the present study does not indicate that these phenotypes are virulent. As, the *CAP59* gene is not the only determinant responsible for the formation of capsules; three other associated capsule genes (*CAP10, CAP60, CAP64*) have been shown to be important for the development of *Cryptococcus* capsules (Okabayashi *et al*., 2007).

In fact, even though most of the identified reported *Cryptococcus* phenotypes were uncapsulated, the awareness of these forms is an important consideration, particularly in immunocompetent hosts which can display unusual courses and challenge timely diagnosis.

Furthermore, Sorrell (2001) study confirmed that the virulence of the fungus and severity of infection is the sum of the route of infection, the other variables such as the *C. gattii* serotype, pathogenic infectious dose, and host immune status.

CONCLUSION

Current results indicate that *Cryptococcus* species other than *C. neoformans* may colonize nasal vestibule of asymptomatic donkeys. The low prevalence of *C. neoformans* indicated limited environmental existence of these fungi in the areas examined. *C. gattii* is common in nature, and its existence in the nasal passages of donkeys suggests that there might be suitable niches for the environmental development of this species in the areas studied. Furthermore, this reinforces the hypothesis that changes in *Cryptococcus* host preferences can be continuous. In order to clarify the epidemiology of this fungus in donkeys and strengthen therapeutic and control approaches, more research to examine the main risk factors of these pathogens should be considered.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20201210211243

Statement of conflict of interest

The authors have declared no conflict of interests.

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