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EMERGENCE OF *CRYPTOCOCCUS SPP*. IN DONKEYS IN EGYPT AND ITS ZOONOTIC POTENTIAL

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ABSTRACT

Over the last decade, Cryptococcus has gained a medical importance, as it becomes an emerging pathogen of immune-competent individuals. Despite, the worldwide geographical expansion of *Cryptococcus* spp. and the changes in its host preference, there are no epidemiological data regarding the occurrence of these fungi in donkeys. The current study was carried out to investigate the possible role of donkeys in the epidemiology of such disease in Egypt. Bacteriological isolation and identification of nasal swabs gathered from 52 diseased and healthy donkeys at different localities in Egypt evidenced that, the overall occurrence of *Cryptococcus* spp. Was 11.5%.The highest percent was recorded in El-Fayoum Governorate (25) followed by Cairo Governorate (10).The lowest percent of *Cryptococcus* spp. was recorded in Giza Governorate. Phenotypic identification of *Cryptococcus* spp. among healthy and diseased donkeys indicated that 13.2 % and 7.1% of the examined donkeys were positive for this pathogen, respectively. The study of the potential risk factors (age, gender, and the health condition) related to the colonization of Cryptococcus in the examined animals revealed no statistically significant differences. Molecular serotyping of six identified *Cryptococcus* spp. evidenced that *C.gattii* was isolated from the nasal passages of four healthy examined donkeys (7.7%); while the other two isolates of *C.neoformans* serotype A (3.8%) were identified in healthy and diseased donkeys**.** Among the 6 positive donkeys, clinical condition was recorded in only one 12 year-old male donkey (16.7%) with stomatitis. To our best knowledge, this is the first study that investigates the emergence of *Cryptococcus* spp. in donkeys, it also highlights a possible association of those fungi with human disease in Egypt. Accordingly, the knowledge obtained from the current study provides a useful tool to start anew epidemiological surveys.

Further investigations of the virulence of those pathogens should also be considered, in order to improve current therapeutic and control strategies.

Keywords:

Donkeys, *C.neoformans*, *C.gattii*, nasal swabs, potential risk factors.

INTRODUCTION

Recently,fungal pathogens as *Cryptococcus* spp. were increasingly recognized as a significant threat to the populations' health all over the world. There are at least 37 different *Cryptococcus* spp. of which; two are important human pathogens *Cryptococcus neoformans* and *Cryptococcus gattii* **(Kwon-Chung *et al.*, 2017)**.The taxonomical classification of *C.neoformans* evidenced *C.neoformans* and *C.deneoformans*, as *C.neoformans* serotype A with three genotypes VNI,VNII,and VNB; and *C.deneoformans* serotype D&genotype VNIV**.** While, in case of *C. gattii*, there are five cryptic species with serotypes B,C and genotype from VGI to IV **(Hagen *et al.*, 2015)**.Although all the serotypes might differ in their geographical spread, they can all cause disease in humans and animals.

Annually, one million cases of cryptococcal meningitis were estimated among people with HIV/AIDS attributed to infection with these species, resulting in nearly 625,000 deaths (Centers for Disease Control and Prevention, CDC, Atlanta, USA, http://www.cdc.gov/).

As well, *C. gattii* appears to have a greater propensity to infect immune-competent humans **(Rozenbaum and Gonçalves, 1994, Speed and Dunt, 1995**).

The infection is likely acquired from the environment by inhalation of spores or dehydrated yeast cells that are able to penetrate the pulmonary alveoli and then disseminate through the bloodstream causing soft tissue infections, pneumonia and most often meningoencephalitis **(Kwon-Chung*et al*., 2014).**

As a consequence of the environmental changes, the number of fungal diseases has increased in animals and plants **(Fisher *et al.*, 2012)**. *C. neoformans* was not only isolated from avian excreta but also from soil and house dust **(Litvintseva *et al*., 2011, Irokanulo *et al*., 1997, Swinne *et al*., 1986**) as well as exotic, migratory birds, domestic and wild animals can be carriers or susceptible hosts for this species **(Casadevall and Perfect. 1998)**. In addition, the plethora of tree species may be colonized by *C. gattii* species complex **(Velez and Escandón. 2017).**Environmental surveys carried out in eight African countries including

Egypt revealed that these pathogens represented 1% of the total reported isolates in environment **(Cogliati M. 2013).**

Food and Agriculture Organization (FAO) last statistics estimates that there are about 3.3 million donkeys (Equusasinus) in Egypt. A vast majority of these donkeys are daily working animals and they form the country's second largest population of livestock after goats. Similar to other mammals, donkeys and horses may be affected with several fungal diseases that represent a serious threat to them as reported by **Fisher *et al*. (2012).** Cryptococcosis in horses is associated mainly with lesions in the respiratory tract, central nervous system (CNS), and abortion. However, disseminated cryptococcosis is reported in horses **(Zoppa *et al*., 2008),** while cutaneous cryptococcosis was recorded in donkeys **(Khodakaram-Tafti and Dehghani, 2006)**.

From the one health concept, the health of these working animals is closely related with the health of the human population. So, there is an urgent demand to investigate the role of donkeys in cryptococcal epidemiological cycle in Egypt. As there is no epidemiological data are available among cryptococcal infection in donkeys in Egypt. Therefore, the current study was carried out to investigate the occurrence of *Cryptococcus* species among healthy and diseased donkeys and to assess its serotypes. As well as, underline the potential role of donkeys as carriers and spreaders of these zoonotic fungi.

Materials and methods:

Samples collection and preparation:

A total of 52 nasal swabs were collected from apparently healthy (n=38) and diseased (n=14) donkeys suffering from wounds, mobility impairment, stomatitis, nasal discharge, ocular discharge or abscess. The samples were collected from Cairo, Giza, and El -Fayoum Governorates. The nasal swabs were taken using sterile bacteriological swabs under complete aseptic condition. The swabs were inserted into both nasal vestibules and rotated vigorously, then inoculated into sterile Sabouraud dextrose broth (Oxoid) supplemented with chloramphenicol (0.1g/L) (Hi Media). The samples were transported in ice box to the laboratory. Data were collected from each individual animal included age, gender, as well as underlying health problems.

Protocol of samples collection was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine, Cairo University, Egypt.

Isolation and phenotypic identification of *Cryptococcus* spp.

Briefly, the inoculated swab samples were incubated at 37°C for 24 h according to **Horta *et al*.(2002).** The supernatant of the prepared sample was streaked onto plates of SDA with chloramphenicol, and then incubated at 37°C for 48-72 h.Seventy-two hours after incubation; colonies with a mucoid appearance were selected and identified by microscopic morphology of yeast cells.

Identification of *Cryptococcus* isolates were performed based on melanin synthesis (brown colour effect). Briefly, a loopful from the original broth were streaked onto plates of Tobacoo agar media (TAM) and incubated at37°C for 3-5 days **(Tendolkar *et al*., 2003 and Refai *et al*.,2005).**

Colonies conforming to cryptococcal morphology were identified and confirmed by RapID yeast plus system (RYP) (Remel, USA) which is a qualitative micro-method employing conventional and chromogenic substrates for the identification of medically important yeast, yeast-like, and related organisms isolated from clinical specimens **(Smith *et al*., 1999and Soltani *et al*., 2013).**

Molecular identification:

Extraction of the Genomic DNA:

Genomic DNA was extracted from the pure Cryptococcus isolates using boiling method according to **Mohammadi *et al*.,(2017)**, the extracted DNA was stored at-20 °C until further use.

Molecular subtyping of *Cryptococcus* spp. Isolates:

Multiplex PCR was carried out using specific oligonucleotide primers displayed in (Table 1) to detect *C. neoformans* serotype A and *C.gattii* serotype B. The PCR reactions were carried out in a total volume of 25µl, containing 3µl of template DNA from each isolate, 12.5 µl of Taq Master Mix (Thermo Scientific), 0.5µl of each primer (Metabion, Germany) and 7.5µl of PCR grade water .The amplification condition was as follows: initial denaturation at 94°C for 8 min, 35cycles each consisting of denaturation at 94°Cfor 1 min, annealing at 65 °C for 1 min, elongation at 72°C for 2 min, and final extension step at 72 °C for 8 min. The PCR amplicons were electrophoresed on agarose gel (1.5 %) at 100 V for 60 min and visualized under ultraviolet light.

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 larger than 2x2) were used. A*P*-value less than 0.05 was considered as significant.

RESULTS

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 percent was recorded in El-Fayoum Governorate (25) followed by Cairo Governorate (10). The lowest percent of *Cryptococcus* spp. was recorded in Giza Governorate (8.8) as shown in (Table 2). The statistical analysis revealed that there is no significant difference (P = 0.363, Fisher's exact test) among the examined localities. Regards to the age and gender of the examined donkeys the highest % of occurrence of *Cryptococcus* spp. was in donkeys of >10 year old (14.1), as well as the occurrence of the pathogen was nearly similar in both of the examined males (11.4%) and females (11.8%) (Table3). Phenotypic identification of *Cryptococcus* spp. among healthy and diseased donkeys evidenced 13.2 and 7.1% of the examined donkeys were positive respectively for this pathogen (Table 4).

Investigation of the potential risk actors (age, gender and the health condition) related to the colonization of Cryptococcus in the examined animals evidenced that there is no statistically significant differences (P = 1.000, Fisher's exact test). 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 isolates of *C.neoformans* A (3.8%) were identified in healthy and diseased donkeys (Table 4).

Among the 6 positive donkeys, clinical condition was recorded in only one 12 year old male donkey (16.7%) with stomatitis.

DISCUSSION

Over the last two decades, the numbers of fungal and fungal-like diseases of plants and animals in both natural and controlled systems have increased, most likely as a consequence

of the environmental changes **(Fisher *et al*., 2012**). As well as, progressive increase in the number of debilitated individuals.

A major number of literature focuses on individual clinical cases, while less is known about the epidemiology of the disease in horses **(Duncan *et al*., 2011)**.

To our knowledge, the role of donkeys in epidemiology of this pathogen has not been specifically investigated. 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 (**Duncan *et al*., 2006 (a&b), Duncan *et al*., 2005, Raso, *et al*. 2004, Krockenberger, *et al*., 2003**).

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%).

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

Our findings demonstrated 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. In this context, **Connolly *et al*., (1999) and Malik *et al*., (1997)** declared that cryptococcus environmental exposure and asymptomatic colonization of the respiratory tract has been proposed to be 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).As well, **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 identified individuals remaining asymptomatic.

C. gattii has recently come to public attention because of an outbreak of devastating illness in immunocompetent individuals. The first case of *Cryptococcal neoformans* var. *gattii* serotype (B) from Egypt was detected in an HIV patient **(Mansour *et al.*, 2006)**. Also, this serotype was included as one of the possible primary agents of granulomatous rhinitis in horses (**Cruz *et al.*, 2017)**. The determination of the species was important because infections by *C. gattii* are increasingly considered worrisome since this species is not susceptible to the most commonly used antifungal agents, which makes the treatment more difficult **(Trilles *et al.*, 2004)**, as well as this pathogen infects immune-competent hosts, especially children .

Age, gender, and health conditions of the individual animals have no statistically significant effect on nasal colonization with *Cryptococcus* spp. as shown in the present study. Study; this might argue for the presence of other risk factors like the environment. Determination of such possible factors can help animal-owners and veterinarians to mitigate the risk of infection with *Cryptococcus* spp.

In conclusion, the current results indicate that *Cryptococcus* species other than *C. neoformans* can colonize the nasal vestibule of asymptomatic donkeys. The low prevalence of *C. neoformans* suggested a limited environmental presence of these fungi in the studied areas. *C.gattii* wide spreading in nature, their occurrence in the nasal passages of donkeys indicates that favourable environmental niches for development of this species are probably present in the studied areas. Furthermore, this reinforces the hypothesis that changes in the host preferences of cryptococcus might be ongoing. Further studies to investigate the virulence of these pathogens should be considered, in order to understand the epidemiology of it in donkeys and improve current therapeutic and control strategies.

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Table (1): Sequence of oligonucleotide primers for molecular serotyping of *C.neoformans* and *C. gattii*

Target agent and Genes	Primer sequence (5'- 3')	Amplicon size (bp)	References
C. neoformans **CNa-70S**	**(ATTGCGTCCACCAAGGAGCTC)**	**695**	**(Aoki *et al.*, 1999, Leal *et al.*, 2008).**
CNa-70A	**(ATTGCGTCCATGTTACG TGGC)**		
C .gattii **CNb-49S**	**(ATTGCGTCCAAGGTGTTGTTG)**	**448**	
CNb-49A	**(ATTGCGTCCATCCA ACCGTTATC)**		

Table (2): Occurrence of *Cryptococcus* spp. in donkeys originated from different localities.

Location	Number of samples	Number 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 (3): Occurrence of *Cryptococcus* spp. according to the age and gender of the examined donkeys.

Predisposing factors		Number of samples	Number of positive samples	% of positive samples
Age (year)	1-5	33	4	12.1
	6-10	12	1	8.3
	>10	7	1	14.3
Total		52	6	11.5
Gender	Male	35	4	11.4
	Female	17	2	11.8
Total		52	6	11.5

Table (4): Occurrence of *Cryptococcus* spp. among healthy and diseased donkeys.

Underlying health condition	Number of samples	positive samples		*Cryptococcus spp.*			
				C.neoformans (A)		*C.gattii (B)*	
		No.	%	No.	%	No.	%
Healthy	38	5	13.2	1	2.6	4	10.5
Diseased	14	1	7.1	1	7.1	0	0.0
Total	52	6	11.5	2	3.8	4	7.7