Horse: a potential source of Cryptococcus neoformans and Cryptococcus gattii in Egypt

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Abstract

Background: Cryptococcosis is an opportunistic mycozoonosis of global significance in a wide variety of host species. In equines, cryptococcosis is uncommon, and sporadic cases have been reported with rhinitis, sinusitis, pneumonia, and meningitis. Cryptococcus spp. represents a potential risk for immunosuppressed and healthy persons. In Egypt, epidemiological data on cryptococcal infection in horses are limited. The current study was carried out to investigate the occurrence of Cryptococcus spp. in horses and its possible role in the epidemiology of such disease in Egypt.

A total of 223 samples was collected from different localities in Egypt included 183 nasal swabs from horses, 28 nasal swabs from humans, and 12 soil samples. Bacteriological examination and the identification of Cryptococcus spp. were performed. Molecular serotyping of Cryptococcus spp. was determined by multiplex PCR using CNa-70S/A-CNb-49S/A. The virulence genes (LAC1, CAP59, and PLB1) of the identified isolates were detected by PCR. Moreover, sequencing and phylogenetic analysis of the C. gattii gene from horses, humans, and soil isolates found nearby were performed.

Result: The overall occurrence of Cryptococcus spp. in horses were 9.3, 25, and 10.7% in horses, the soil, and humans, respectively. Molecular serotyping of the Cryptococcus spp. isolates recovered from the nasal passages of horses proved that C. gattii (B), C. neoformans, and two hybrids between C. neoformans (A) and C. gattii (B) were identified. Meanwhile, in case of soil samples, the isolates were identified as C. gattii (B). The human isolates were serotyped as C. gattii in two isolates and C. neoformans in only one isolate. Molecular detection of some virulence genes (LAC1), (CAP59), and (PLB1) were identified in both C. gattii and C. neoformans isolates. The C. gattii gene amplicons of the isolates from horses, humans, and the soil were closely related.

Conclusion: This study provides the first insights into the Egyptian horse ecology of Cryptococcus species and highlights the role of horses as asymptomatic carriers in disseminating the potentially pathogenic Cryptococcus spp. It also presents the possible risk of cryptococcosis infection in humans.

Keywords: C. neoformans, C. gattii, Horse, Serotyping, Egypt

Background

Cryptococcosis is a life-threatening systemic mycosis of global significance that affects a wide variety of host species. It is caused by the Cryptococcus neoformans species complex and the Cryptococcus gattii species complex and is usually associated with pulmonary and systemic infections in humans and animals [1, 2].

The C. neoformans species complex includes two separate species, C. neoformans (serotype A) represented by genotypes VNI, VNII, and VNB and C. deneoformans (serotype D) represented by genotype VNIV. The C. gattii species complex has five cryptic species with serotypes B and C and genotype from VGI to V. Moreover, interspecies hybrids between C. neoformans and C. gattii have been detected [3–5]. C. neoformans (A) is found...
and the production of degradable enzymes such as phospholipase B (PLB1) [12]. These virulence factors confer a selective advantage to C. neoformans that enables it to reside both in the environment and in mammalian hosts.

C. neoformans is the most common fungal pathogen that infects the human central nervous system [13]. C. neoformans causes life-threatening infections as meningocencephalitis primarily in immunocompromised hosts (immunodeficiency generally associated with AIDS) [14]. Unlike C. neoformans, C. gattii causes meningocencephalitis and pulmonary infections mainly in immunocompetent hosts.

In horses, the distribution of cryptococcosis is worldwide, whereas the majority of clinical infections are reported from Western Australia and Vancouver Island, British Columbia, Canada [8, 15]. Moreover, a fatal case of a disseminated C. gattii infection was identified in an Arabian horse imported from South Africa into the United Arab Emirates and identified as C. gattii VGII, which was closely related to another C. gattii VGII isolate from the Middle East [16].

In Egypt, most studies at the beginning of the year 2003 reported that the major subtype of the C. neoformans spp. complex isolated from either environmental (Plant or Bird origin) or clinical samples (buffaloes, cattle, sheep, and chicken) is C. neoformans (serotype A) [17, 18]. Besides, Elhariri et al. [19] revealed the identification of C. neoformans and C. gattii from pigeon dropping. Furthermore, plants from Qutur and Tanta area carried C. gattii [20]. Abdel-Salam [21] reported on the characterization of Cryptococcus serotypes A and A/D in clinical specimens (serum, CSF, and stool samples). But to date, little epidemiological data are available among cryptococcal infection in horses and humans in contact in Egypt.

Therefore, the current study was carried out to determine the prevalence of Cryptococcus spp. among horses and the role played by horses in the distribution of such pathogens in the surrounding environment and humans occupationally in contact with such animals.

Methods

Sample collection and preparation

A total of 223 samples were collected from different localities in Egypt (The Cairo, El-Fayoum, Qalyubia, and Giza Governorates) over a period of 1 year from December 2018 to December 2019. These samples included 183 nasal swabs from horses and 28 nasal swabs from humans. In addition, 12 soil samples were collected from the houses of horses. Data collected from each human and animal included age, gender, and underlying health problems.

The nasal swabs were taken using sterile bacteriological swabs under complete antiseptic conditions and inserted into both nasal vestibules and rotated vigorously. The collected samples were immediately transported in ice boxes to the laboratory and then inoculated into sterile Sabouraud dextrose broth (Oxoid) supplemented with chloramphenicol (0.1 g/L) (HiMedia).

Soil samples were collected in sterile plastic bags. The samples were prepared by mixing about 3–5 g of each sample into sterile test tubes containing 10 ml of Sabouraud dextrose broth supplemented with chloramphenicol. The tubes were shaken vigorously by vortex and allowed to stand for about 15 min [18].

Isolation and phenotypic identification of Cryptococcus spp.

The inoculated swab samples of humans and horses in addition to the prepared soil samples were incubated at 37°C for 24 h according to Horta et al. [22]. The supernatant of the incubated sample was streaked onto plates of SDA with chloramphenicol and then incubated at 37°C for 48–72 h. The typical Cryptococcus colonies with a mucoid appearance were selected and identified by the microscopic morphology of yeast cells.

Cryptococcus isolates were confirmed based on melanin synthesis (brown color effect) by streaking on the tobacco agar plates [23]. The identification of Cryptococcus isolates was done using the Rapide yeast plus system (Remel, USA) [24].
Extraction of the genomic DNA
Genomic DNA from the pure Cryptococcus isolates was extracted using the boiling method according to Mohammadi et al. [25]. The extracted DNA was stored at −20 °C until further use.

Molecular serotyping of Cryptococcus spp. isolates
Multiplex PCR was carried out to detect C. neoformans serotype A and C. gattii serotype B as previously described by Aoki et al. [26] using specific oligonucleotide primers set. PCR was carried out on a total volume of 25 μl, containing 3 μl of template DNA from each isolate, 12.5 μl of EmeraldAmp MAX PCR Master Mix (Takara, Japan), 0.5 μl of each primer (10 pmole/μl; Metabion, Germany), and PCR-grade water, all of which added up to 25 μl. The PCR products were visualized on 1.5% agarose gel.

Molecular detection of the virulence genes (LAC1, CAP59, and PLB1)
Conventional PCR to amplify the LAC1, CAP59, and PLB1 genes was performed according to the method of Meyer et al. [27]. Briefly, the amplifications were carried out in a total volume of 25 μl, containing 3 μl of template DNA from each isolate, 12.5 μl of EmeraldAmp MAX PCR Master Mix (Takara, Japan), 0.5 μl of each primer (1 μM) (Metabion, Germany), and completed up to 25 μl by PCR-grade water. The PCR amplicons of the three genes were electrophoresed on 1.5% agarose gel.

Sequencing and sequence analysis
The amplification products of three C. gattii isolates from horses, humans, and the soil were selected and purified using the QIAquick gel extraction kit (QIAGEN, Germany) according to the manufacturer’s instructions and sequenced at the Animal Health Research Institute (Giza, Egypt) using the forward and reverse primers of C. gattii (CNb-49s and CNb-9A).

The nucleotide sequences have been deposited in the National Center for Biotechnology Information (NCBI) GenBank database under the accession numbers MT520167-MT520169.

The nucleotide sequences of C. gattii isolates were compared with the sequences available in the public domain using the NCBI BLAST server. Publicly available gene sequences were retrieved from the NCBI GenBank and aligned using CLUSTALW in BioEdit version 7.0.1.4. Phylogenetic analysis was performed with MEGA version X using the neighbor-joining approach. The bootstrap consensus tree was estimated from 1000 replicates.

Results
The overall occurrence of Cryptococcus spp. collected from 183 nasal swabs of horses at different localities in Egypt was 17/183 (9.3%). Molecular serotyping of the Cryptococcus spp. isolates recovered from the nasal passage of horses proved that C. gattii was identified in 12 isolates and C. neoformans A in 3 isolates. Two hybrids between C. neoformans A and C. gattii B were also identified. In the case of soil samples, C. gattii B was detected in 3 out of 12 samples (25%) collected from horse houses. The human isolates were serotyped as C. gattii in two isolates (7.1%) and C. neoformans in only one isolate (3.6%).

The detection rates of Cryptococcus spp. in healthy horses showed that C. gattii was identified in 5 out of 44 samples (11.4%). In the case of diseased horses, the detection rate was 12/139 (8.6%), and the identified Cryptococcus spp. in each case mentioned in (Table 1). The occurrence of the Cryptococcus spp. was nearly similar in both males and females as shown in Table 1.

Regarding the human samples, Cryptococcus spp. were identified from 3 out of 28 samples (10.7%), and C.

Table 1 Occurrence of Cryptococcus spp. in horses according to health condition and gender

| Predisposing factors | No of isolates | Cryptococcus spp identified | % of positive isolates |
|----------------------|---------------|----------------------------|-----------------------|
| Health condition     |               |                            |                       |
| Healthy              | 5             | C. gattii (5)              | 11.4 (5/44)           |
| Diseased             | 12            | C. gattii (5) C. neoformans (1) | 8.6 (12/139)          |
| Respiratory tract signs (6) |           | C. gattii (2) C. gattii & C. neoformans hybrids (2) |           |
| Nervous Signs (4)    |               |                            |                       |
| Wounds treated with long course of antibiotics (2) |           |                            |                       |
| Gender               |               |                            |                       |
| Male                 | 11            | C. gattii (8) C. neoformans (1) C. gattii & C. neoformans hybrids (2) | 9.5 (11/116)          |
| Female               | 6             | C. gattii (4) C. neoformans (2) | 9 (6/67)              |
| Total                | 17            |                            | 9.3 (17/183)          |
neoformans serotype (A) was isolated from the nasal passage of an immune-compromised male suffering from chest allergy. In addition, C. gattii was isolated from two asymptomatic individuals. The positive samples belonged to humans working in stud farms where the horses’ positive samples were collected. Our results indicated that Cryptococcus spp. was more prevalent in old male individuals. Moreover, we found that Cryptococcus spp. more frequently colonized the nasal passages of smokers than those of non-smokers as shown in Table 2.

The molecular detection of some virulence genes LAC1, CAP59, and PLB1 was carried out in both C. gattii and C. neoformans isolates (Table 3). Comparing the partial sequences of C. gattii B revealed homology between the three selected isolates from horses, the soil, and humans in the same vicinity (Fig. 1).

**Discussion**

The published papers clarified the problem of mycoses in horses, including pathogenic fungi, from different parts of the world [28]. Among the mycotic diseases of horses,
Cryptococcosis is of particular concern [29]. Egypt is one of the countries widely known for breeding Arabian horses for the local and foreign markets. In addition, the horse populations in Egypt are widely used in tourism [30]. However, up to date, there have not been enough epidemiological data in Egypt for cryptococcal infection in horses.

In the current study, the overall recorded prevalence of Cryptococcus spp. colonization detected in the nasal passages of the examined horses was 9.3%, with the value being higher (11.4%) in healthy examined horses, which probably indicates that apparently healthy horses could be asymptomatic carriers of Cryptococcus spp. These findings are supported by those of Malik et al. [31], Connolly et al. [32], Duncan et al. [33], and Mohamed et al. [34], who isolated Cryptococcus spp. from the nasal passages of dogs, cats, koalas, and donkeys without any evidence of disease, suggesting the asymptomatic colonization of the nasal mucosa following environmental exposure.

The species identification of Cryptococcus appeared to be higher with C. gattii (12 isolates) in examined horses than with C. neoformans (3 isolates). This result was similar to that of McGill et al. [15], who detected Cryptococcus spp. from 20 out of 155 affected horses in Western Australia with a high frequency of C. gattii, and Duncan et al. [8], who identified C. gattii in the nasal passage of horses in Canada.

Hybrids between C. neoformans (A) and C. gattii (B) were also identified in two isolates, which is in line with the findings of Bovers et al. [35] and Aminnejad et al. [4], who reported that C. gattii is capable of forming hybrids with C. neoformans. Interspecific cryptococcal hybrids have been reported earlier in Germany [36], Denmark [37], and the United States [38]. Inter-varietal and interspecies hybrids of Cryptococcus are more virulent and cause mammalian diseases [39]. This hybrid might become a “superpathogen,” which has a worldwide distribution like C. neoformans and is able to infect immunocompetent humans like C. gattii, combining the malicious characteristics of both species [35].

The pathogenic species C. neoformans and C. gattii infect immune-suppressed and immune-competent individuals, respectively [1, 40]. However, the data on the asymptomatic isolation of Cryptococcus spp. from human nasal passages are very rare. In this study, we isolated Cryptococcus spp. from people without cryptococcosis, Morera et al. [41] supported similar findings of the asymptomatic nasal carriage of C. gattii in humans sharing the same house with domestic ferret having cryptococcosis.

The occurrence of Cryptococcus spp. in the nasal passage of humans who have occupational contact with infected horses was 10.7% (3 out 28); two of them were reported in aged individuals who were smokers, which is in line with the findings of Forthal et al. [42] and Pourbaix et al. [43], who reported that smoking was a risk factor for invasive fungal diseases such as cryptococcosis.

In this study, the C. neoformans serotype A was identified from the nasal passage of an immune-compromised male suffering from chest allergy. In addition, the C. gattii isolate was isolated from two asymptomatic individuals in contact with horses. In this context, a clinical isolate was obtained from an immigrant worker from Kuwait that is closely related to a case of C. gattii in an Arabian horse in the United Arab Emirates [16].

The epidemiological significance of this research lies in its proving that both horses and humans are at risk of environmental contamination in stud farms. In these farms, eucalyptus trees are used for horse shelter and shadowing [44], and these trees are one of the sources of C. gattii. Besides, the pigeon is a natural reservoir of C. neoformans, and it usually eats the remains of equine food (e.g., seeds and grains) from the feed troughs and defecates in the feed and water trough.

To support our epidemiological view, the soil around horses included in this research was examined for Cryptococcus spp. and the result revealed 25% from which all isolates were C. gattii. This may be attributed to the fact that soil contaminated with plant debris and decaying wood is a major environmental source of C. gattii [7].

The isolation of C. gattii from the nasal passage of horses, humans, and soil samples in close vicinity to the examined horses point to the presence of a common issue between these isolates, which was predicted to be the excessive presence of eucalyptus trees in the collection sites. This result is in line with that of McGill et al. [15], who reported that horses were more likely to be infected with C. gattii than with C. neoformans, and this was attributed to horse population’s close association with eucalyptus trees, a regional environmental reservoir of C. gattii.

Human cryptococcosis develops following environmental exposure and inhalation of the infectious spores of Cryptococcus spp. This is enhanced by some virulent determinants, which are enzyme secretion, the addition of melanin to the fungal cell wall, and the abundant secretion of polysaccharides that may surround the cell to form a capsular structure [45].

In this study, we investigated the presence of some virulence genes such as laccase (LAC1), capsular-associated gene (CAP59), and phospholipase (PLB1) in the identified Cryptococcus spp. isolates. The International Society of Human and Animal Mycology (ISHAM) group selected the Multilocus Sequence Type (MLST) analysis as the method of choice for global molecular epidemiological
typing of Cryptococcus species using seven housekeeping loci (CAP59, GDP1, LAC1, PLB1, SOD1, URA5, and IGS1) [27]. The study identified the laccase (LAC1) gene, which is the main protein involved in the production of the melanin pigment, in all C. neoformans and C. gattii isolates [46, 47]. The study also demonstrates the presence of the CAPS9 gene in the identified isolates. This capsular gene has a vital role in Cryptococcus virulence.

Robertson et al. [48] demonstrated that strains producing large capsules were more likely be associated with higher intracranial pressures and lower fungal clearance rates upon treatment with amphotericin B and fluconazole in HIV-infected patients. Furthermore, we sequenced the phospholipase (PLB1) gene (which is an important gene in maintaining the integrity of the chitinous fungal cell wall as well as providing nutrients that can be utilized as a carbon source for C. neoformans during the course of the infection) in all isolates [49].

The studied virulence genes have been generally found to be recovered from the horses, horses’ environments, and humans in contact with the horses, which is in line with the findings of Firacative et al. [50], who supports the theory of the acquisition of the infection from environmental sources. Likewise, Esher et al. [51] hypothesized that the virulence traits were acquired for survival in the environment and then re-purposed in the setting of mammalian infection.

In the present study, a phylogenetic relationship was required to predict the epidemiological infection cycle of Cryptococcus. C. gattii sequenced from different sources (humans, horses, and the soil) confirmed that it can be isolated from those three sources with the same sequence type and indicated that C. gattii was shared between humans, animals, and the environment. The phylogenetic tree showed that the three sequences were found on the same clade, which explains the probability of the transmission of the infection between these sources. The presence of eucalyptus trees in equine farms could be one of the environmental sources of infection of horses with C. gattii, which would explain how the people in the surroundings get infected. The same theory was put forward by Farrer et al. [5], who discovered C. gattii VGV in samples from Hyrax-associated environments, which is suggestive of an association with these mammals that can spill over into humans.

**Conclusion**

The present study confirmed the presence of C. neoformans and C. gattii from clinical samples (nasal swab from humans and horses) and C. gattii from environmental samples (soil) with clarifications of the possible epidemiological relationship between environmental and clinical isolates. Further studies need to be carried out on virulence by measuring capsule size and enzyme production. The research results provide insights into the potential exposure risk of humans in contact with cryptococcal infection in the investigated locations.

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