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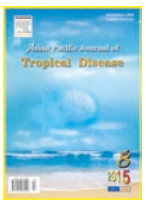

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First report of environmental isolation of *Cryptococcus neoformans* and other fungi from pigeon droppings in Makkah, Saudi Arabia and *in vitro* susceptibility testing

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ABSTRACT

Objective: To verify the occurrence of *Cryptococcus neoformans* (*C. neoformans*) and other fungi in samples of pigeon droppings collected from Makkah city, Saudi Arabia.
Methods: One hundred and twelve withered pigeon dropping samples were collected from 12 different districts. Using the dilution plate technique, samples were cultured on Sabouraud dextrose agar and esculin agar. Colonies were examined microscopically and *C. neoformans* identification is confirmed by India ink preparation, observation of urease activity and brown pigmentation on esculin medium. Susceptibility patterns of five yeast species and four molds against five antifungal drugs were tested using agar disk diffusion method.
Results: *C. neoformans* was recovered from 38 samples (34%). Na'aman valley was recorded to be the highest contaminated site (66.7%) with *C. neoformans*, while the samples collected from Al Awaly district were considered as the lowest contaminated samples (6.7%). Also, twenty species related to sixteen genera of fungi other than *C. neoformans* were recovered from which, three yeast genera were recorded. The antifungal susceptibility testing showed that the nine tested fungal species were sensitive to Mycosat, while Fungican exerted inhibition zones of four species only. *C. neoformans* was moderately sensitive towards all tested compounds but it can resist Flucoral where no inhibition zone could be detected.
Conclusions: Our results are considered to be the first report on the environmental prevalence of *C. neoformans* in pigeon feces in Makkah, Saudi Arabia. The data indicated that pigeon droppings can be considered as a potential source of this basidiomycetous yeast in addition to other fungal species in this region.

1. Introduction

Makkah is located in a narrow valley at a height of 277 m above sea level (Figure 1a). The resident population in 2012 was roughly 7.47 million representing 26% of the total Saudi Arabian population[1]. Visitors to Makkah can reach more than 7 million every year. Makkah is characterized by the existence of a large number of free-living pigeon flocks in many sites of the city. In general, wild free-living birds (*e.g.* pigeons) are regarded as visible indicators of diverse and healthy environments. Yet, from a public health standpoint, this positive view may not always be valid, since free-living birds carry a diversity of microorganisms that are pathogenic to humans[2]. Pigeon droppings have been reported as the major environmental source of *Cryptococcus neoformans* (*C. neoformans*) as well as many other pathogens in several countries[3-5]. *C. neoformans* is an encapsulated yeast-like fungus that causes cryptococcosis and its inhalation from environmental sources may cause pulmonary and neurological diseases in susceptible humans[6]. The incidence of cryptococcal infection has recently increased around the world as a result of a large increase in patients suffering from AIDS, population aging, and the expanded use of immunosuppressive drugs for cancer treatment and organ transplantation[6-8]. Since there are no available studies on the environmental distribution of *C. neoformans* in Saudi Arabia, there is an urgent need to investigate this point. The present study

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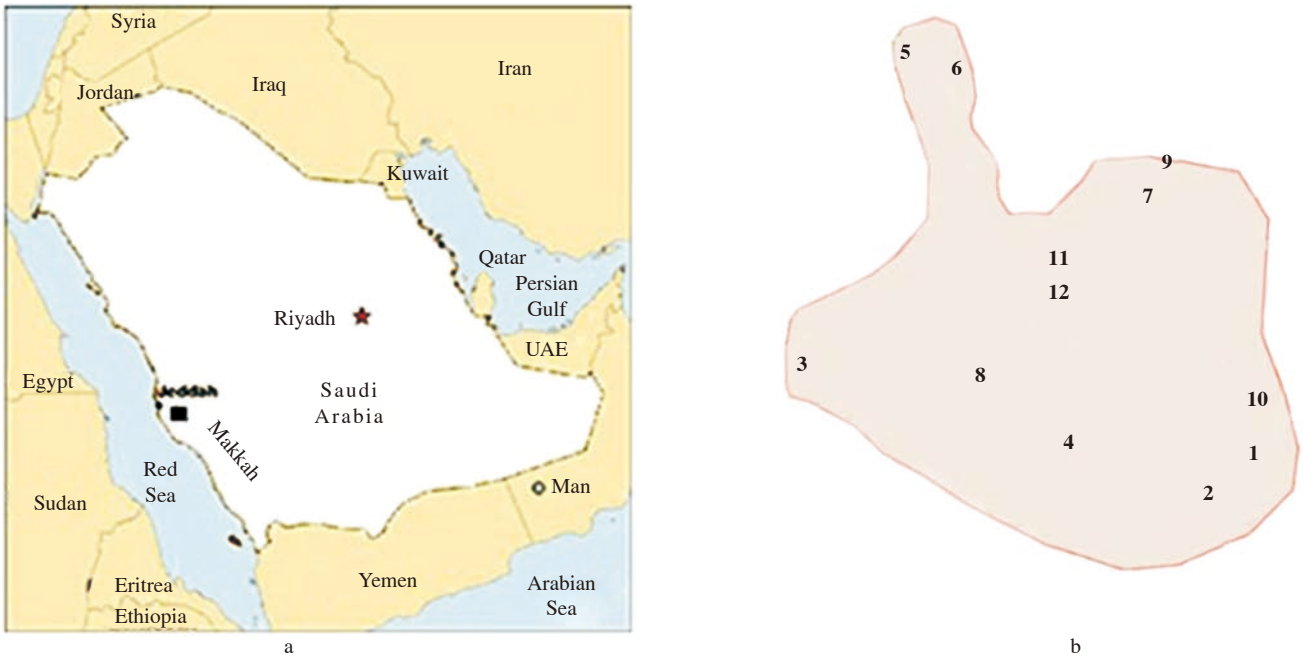

Figure 1. Saudi Arabian map showing geographical location of Makkah (a) and sampling sites in Makkah (b).
1: Al-Abedia; 2: Al-Awaly; 3:Al-Hamraa; 4: Al-Hegra; 5: Al-Nawaria; 6: Al-Omra; 7: Al-Sharayie; 8: Ashawquia; 9: Gorana; 10: Na'aman valley; 11: North Aziziya; 12: South Aziziya.

can be considered as the first step that aimed to know the extent of existence and environmental distribution of *C. neoformans* in pigeon droppings in Makkah that is considered as the highest populated city in Saudi Arabia.

2. Materials and methods

2.1. Collection of samples

Total of one hundred and twelve samples of withered pigeon droppings were collected from 12 different sites within the city (Figure 1b), over a period of six months (July to December, 2014). The samples were placed in sterile universal bottles and processed in the same day of sampling.

2.2. Isolation and identification of C. neoformans and other fungi

The method described by Xavier *et al.* was adapted as follows: About 10 g of withered pigeon droppings from each site were transferred to Erlenmeyer flasks containing a saline solution (0.9%) with chloramphenicol (200 mg/L), achieving 1:10 dilution (w/v) [9]. The mixture was shaken for 20 min and allowed to settle for 30 min. Aliquots of 0.5 mL supernatant were streaked on esculin agar (Oxoid, Basingstoke, UK) for isolation of *C. neoformans* and on Sabouraud dextrose agar (SDA) (Oxoid), for isolation of other fungal species[10]. The plates were then incubated at 37 ℃ for 10 days. Each inoculated plate was examined daily for yeast growth. Colonies with a mucous appetence and those with brown pigmentation on esculin agar (Oxoid) were selected and subcultured on SDA slants to obtain pure cultures. For identification of *C. neoformans*, morphological and biochemical tests were used. The presence of capsule on India ink preparation was considered as one of the bases of *C. neoformans* identification and confirmed by melanin synthesis on esculin agar (Oxoid), urease production on urea agar (Oxoid), and ability to grow at 37 ℃[9,11]. The other fungal species growing on SDA were identified microscopically.

2.3. Antifungal susceptibility testing

Agar disk diffusion method described by Elbanna *et al.* was mainly applied, and five antifungal compounds were purchased from different pharmacies in Makkah city to examine the antifungal susceptibility patterns of *C. neoformans* and other medically important fungal species that were recovered from pigeon feces[12]. These antifungal drugs were: Batrafen (ciclopirox–olamine), Canasten (clotrimazole), Flucoral (fluconazole), Fungican (fluconazole) and Mycosat (nystatin). The tested species were *Aspergillus flavus* (*A. flavus*), *Candida albicans* (*C. albicans*), *C. neoformans*, *Rhizopus stolonifer* (*R. stolonifer*), *Rhodotorula mucilaginosa* (*R. mucilaginosa*), *Syncephalastrum racemosum* (*S. racemosum*), *Trichoderma harzianum* (*T. harzianum*), *Trichosporon asahii* (*T. asahii*) and *Trichosporon mucoides* (*T. mucoides*). Potato dextrose agar plates were inoculated with each tested species by swabbing the fresh cultures onto the surface of agar plates. Sterilized filter paper discs (6 mm) were saturated with 100 μg/mL of each tested antifungal drug and subsequently dried at 40 ℃. The dried disks were transferred to the surface of the inoculated plates in triplicates. Plates were incubated for 36 h at the optimum temperature for each fungal species and their sensitivity to the antifungal compounds was determined by measuring the diameter of inhibition zones (mm). Sterilized water without tested compounds was used as a control.

3. Results

C. neoformans was recovered from 38 out of 112 (34%) pigeon droppings in 11 out of 12 sites examined. Samples collected from site number 3 yielded negative result (Table 1). The highest rate of *C. neoformans* contamination was recorded from the samples collected

from Na'aman valley (site number 10), where 66.7% (four out of six samples) was positive. North Aziziya (site number 11) came second to Na'aman valley regarding *C. neoformans* recovery rate where it was isolated from 9 out of 16 (56.3%) samples. Al-Hegra district came third, where 50% of samples (3 out of 6) were positive (Table 1). *C. neoformans* was also recorded in four other sites (6, 1, 7 and 12) with percentage of contamination as: 40.0%, 37.5%, 33.3% and 33.3%, respectively. The occurrence of *C. neoformans* in samples collected from sites number 5, 8 and 9 were ranged between 20%-25%. On the other hand, samples collected from Al-Awaly district recorded the lowest rate of recovery, where *C. neoformans* was isolated once out of 15 samples representing 6.7% of tested samples (Table 1).

Table 1
Distribution of *C. neoformans* isolated from pigeon droppings in Makkah.

Site of isolation	Site number	Total number of samples	Positive samples	% of positive samples
Al-Abedia	1	16	6	37.5
Al-Awaly	2	15	1	6.7
Al-Hamraa	3	1	0	0.0
Al-Hegra	4	6	3	50.0
Al-Nawaria	5	4	1	25.0
Al-Omra	6	5	2	40.0
Al-Sharayie	7	9	3	33.3
Ashawquia	8	17	4	23.5
Gorana	9	5	1	20.0
Na'aman valley	10	6	4	66.7
North Aziziya	11	16	9	56.3
South Aziziya	12	12	4	33.3
Total		112	38	34.0

The isolation of other yeast and molds species from pigeon fecal droppings was shown in Table 2. Twenty species related to sixteen fungal genera were isolated within the twelve sites that were surveyed. Mucorales were represented by 8 species related to 6 genera from which *R. stolonifer, S. racemosum* and *Mucor hiemalis* were the most common and appeared in 16.1%, 11.6% and 10.7% of the examined samples, respectively. According to the diversity of genera, the genus *Aspergillus* ranked the second to Mucorales isolated from pigeon fecal samples and was represented by five species, namely, *A. flavus, Aspergillus niger* (*A. niger*), *Aspergillus parasiticus, Aspergillus tamarii* and *Aspergillus terreus*. The remaining genera listed in Table 2 were represented either by two or one species. *A. niger* and *R. mucilaginosa* were recorded in high frequency of occurrence and isolated from 43 and 35 pigeon fecal samples representing 38.4% and 31.3%, respectively. *A. flavus, R. stolonifer* and *Saccharomyces* species were encountered in moderate frequency of occurrence. The remaining fungal species were recovered from less than 15% of the samples.

Antifungal susceptibility patterns against five antifungal drugs were shown in Table 3. *C. albicans* and *T. mucoides* were sensitive to all tested antifungal compounds followed by *T. harzianum*, *C. neoformans*, *R. mucilaginosa* and *A. flavus* that were sensitive to 4 out of 5 tested compounds. *S. racemosum* and *T. asahii* were sensitive to three out of five compounds. On the other hand, *R. stolonifer* exhibited the lowest susceptibility patterns to all tested compounds. Also, it was noticed that Mycosat was found to be effective as antifungal compound against all tested isolates, followed by Canasten and Flucoral.

Table 2
Fungal genera and species (other than *C. neoformans*) isolated from pigeon droppings in Makkah.

Fungal genera and species	No. of isolation cases	Frequency of occurrence %
Actinomucor elegans	2	1.8R
Absidia corymbifera	1	0.9R
A. flavus	22	19.6M
A. niger	43	38.4H
Aspergillus parasiticus	13	11.6L
Aspergillus tamarii	3	2.7R
Aspergillus terreus	1	0.9R
C. albicans	14	12.5L
Cunninghamella echinulate	4	3.6R
Mucor hiemalis	12	10.7L
Mucor racemosus	10	8.9L
Papulaspora candida	9	8.0L
Penicillium spp.	7	6.3L
Rhizoctonia solani	4	3.6R
Rhizopus oryzae	2	1.8R
R. stolonifer	18	16.1M
R. mucilaginosa	35	31.3H
Saccharomyces spp.	18	16.1M
S. racemosum	13	11.6L
T. harzianum	1	0.9R
T. asahii	7	6.3L
T. mucoides	3	2.7R
Trichosporon spp.	7	6.3L
Ulocladium sp.	2	1.8R
Sterile mycelia dark	7	6.3L
Total	16 genera and 20 species	

H: High occurrence present in more than 30% of samples; M: Moderate occurrence present in 15% to less than 30%; L: Low occurrence present in 5% to less than 15%; R: Rare occurrence present in less than 5%.

Table 3
Sensitivity of some selected fungal species isolated from pigeon dropping samples to five pharmaceutical antifungal compounds (at concentration of 100 μg/mL).

Tested fungi	Inhibition zone in diameter (mm)				
	Batrafen	Canasten	Flucoral	Fungican	Mycosat
A. flavus	20	10	35	0.0	39
C. albicans	20	11	41	25	32
C. neoformans	35	28	0	27	20
R. stolonifer	0	0	30	0	11
R. mucilaginosa	27	20	60	0	43
S. racemosum	0	11	35	0	36
T. harzianum	0	15	40	10	48
T. asahii	0	11	24	0	35
T. mucoides	24	9	25	22	25

0: Resistant; < 20: Low sensitive; 20 – < 40: Moderately sensitive; ⩾ 40: Highly sensitive.

4. Discussion

The current study showed that *C. neoformans* was found in 34% of 112 samples of pigeon droppings collected from 12 different sites in Makkah. Although the saprophytic distribution of *C. neoformans* in pigeon droppings was previously reported in different countries around the world such as Brazil[13], China[14], Columbia[15], Iran[16], India[9], and Italy[17], information about the environmental existence of this opportunistic fungus in Saudi Arabia is sparse. Currently there is only one published record reporting the incidence of *C. neoformans* infection in a tuberculous lymphadenitis patient in Saudi Arabia[18], therefore, the current study reports for the first time the isolation of environmental *C. neoformans* in Saudi Arabia.

It must be mentioned that, all pigeon dropping samples collected in our study were withered, based on the previous reports indicated that *C. neoformans* was not found in fresh pigeon droppings, probably because dry fecal droppings contain less bacterial flora and thus less competition for nutrients[19,20]. It is generally considered that the pigeon droppings are the main environmental source of *C. neoformans* probably due to its high content of organic material, particularly urea and creatinine[3,5,9,13,14,16,19]. Other environmental sources of the fungus include dust, chicken habitats, fruits and trees[21-23].

In this study, the relatively high rate of *C. neoformans* contamination (> 50%) in three sites (Na'aman valley, Al-Hegra and north Aziziya) could be attributed to the environmental conditions such as large amount of pigeon droppings, dense populated resident and commercial areas, parks and trees, where pigeons may find appropriate locations for nesting and gathering in large numbers. On the other hand, our results showed that *C. neoformans* was recovered once from 15 pigeon fecal samples collected from Al-Awaly district. This can be explained on the basis that this district is relatively newly constructed comparing with the other districts in Makkah resulting relatively lower numbers of human and pigeon population. Thus areas inhabited by large numbers of pigeons flocks may be considered as an important ecological niche for *C. neoformans*[20].

Using the dilution plate method and SDA medium, our data showed that 20 species related to 16 genera of molds and yeasts other than *C. neoformans* were recovered from pigeon droppings. It must be mentioned that, among these 20 molds and yeasts species that were recovered, four species were of medical importance: *C. albicans, R. mucilaginosa, T. asahii* and *T. mucoides*. Costa *et al.* confirmed that urban pigeon droppings are a potential source of pathogenic yeasts and they isolate the same genera from pigeon droppings in Northeast Brazil[13]. *C. albicans* and other *Candida* spp. are commonly found in the gastrointestinal tract, oral cavity, and genital areas as harmless commensals[24]. It is worth noting that as well as being harmless commensals, *Candida* spp. are opportunistic pathogens capable of causing a wide range of superficial, localized, and/or systemic infections[25]. *Trichosporon* species are considered the second most common cause of fungaemia in patients with haematological malignant disease[25]. *Rhodotorula* species are being increasingly recognized as important human pathogens in severely immunosuppressed hosts, especially for patients with advanced HIV infection or cancer who are undergoing transplant[25-28].

In the present study, eight species related to Mucorales and five *Aspergillus* spp. in addition to unidentified *Penicillium* were recorded. Also, a number of species related to the following genera *Penicillium, Aspergillus, Mucor*, and *Rhizopus* were isolated from pigeon dropping samples collected from Isfahan[16]. Some molds recovered during the present study were previously reported to be associated with human diseases such as *Aspergillus* species[29,30]. The Mucorales group (*Mucor* spp., *Rhizopus* spp., Absidia spp., and *Cunninghamella* spp.) were also considered as opportunistic human pathogens[31,32]. One species of *Trichoderma* (*T. harzianum*) recorded once in our study. *T. harzianum* and *Trichoderma longibrachiatum* constitute a lethal hazard for individuals with reduced resistance including patients with leukemia, HIV positive or having transplants[33,34].

The results of antifungal susceptibility testing showed that Mycosat (nystatin) had a broad spectrum antifungal effect where it inhibited all tested fungal species. On the contrary, Fungican (fluconazole) had the narrowest antifungal effect spectrum on tested species, where five of them were resistant. *C. neoformans* showed resistance to Flucoral but was moderately sensitive to other antifungal drugs. The resistance of *C. neoformans* to Flucoral (fluconazole) was in accordance with the finding of some other authors[35]. Varma *et al.* reported heteroresistance of *Cryptococcus gattii* to fluconazole[36]. The *in vitro* antifungal susceptibility studies of isolates of the *C. neoformans*/*Cryptococcus gattii* species complex revealed contradictory results[37]. The study indicated a clear correlation between antifungal susceptibilities and genotypes of the causative cryptococcosis agents. Resistance to antifungal drugs is rare among clinical isolates of *C. neoformans* but has been reported[38]. Despite there is few comparison of minimum inhibitory concentrations data between clinical and environmental isolates, the results obtained by Chowdhary *et al.* showed some differences in the patterns of susceptibility according to the origin of *C. neoformans* isolates (*i.e.* clinical or environmental)[39].

With respect to other fungal genera, our results showed that *A. flavus, R. stolonifer* and *S. racemosum* were resistant to Fungican (fluconazole). The isolates of *A. niger* and *A. flavus* showed varying degrees of resistance to itraconazole, ketaconazole and amphotericin B, resulting in moderate zone of inhibitions against the antifungal agents[40]. In the present study, two yeast species isolates (*C. albicans* and *T. mucoides*) were sensitive to all tested drugs, while *R. mucilaginosa* showed resistance to Fungican. Also, *T. asahii* can resist Fungican in addition to Batrafen. The resistance or sensitivity of some yeast isolates were also noticed by Sardi *et al.* who reported that 62.2% of the *C. albicans* isolates tested were susceptible to fluconazole, 15.6% to voriconazole, 91.1% to amphotericin B and 95.5% to caspofungin[41].

Our results are considered to be the first report on the environmental prevalence of *C. neoformans* in pigeon fecal samples collected from different sites of Makkah, Saudi Arabia. The data indicated that pigeon droppings can be considered as a potential source of this basidiomycetous yeast in addition to other fungal species in this region. Some of the recovered fungal species reported in this study were previously reported as pathogens associated with various types of infections in immunocompromised patients. The possibility of inhalation of the fungal spores by immunocompetent individuals, would probably lead to serious health hazards. Therefore, it is necessary to prevent the accumulations of these large flocks of pigeons for long periods of time in public areas, particularly near residential areas, public parks, food outlets and hospitals. Further studies are required to investigate the environmental distribution of *C. neoformans* and other opportunistic fungi in pigeon droppings in other populated major cities of Saudi Arabia. The *in vitro* susceptibility of nine selected fungal species with medicinal interest isolated from pigeon droppings to five conventional antifungal drugs that recommended for use in Makkah proved that tested fungal species responded variably between resistance and susceptibility.

Conflict of interest statement

We declare that we have no conflict of interest.

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