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ORIGINAL PAPER

Diversity, Virulence Factors, and Antifungal Susceptibility Patterns of Pathogenic and Opportunistic Yeast Species in Rock Pigeon (*Columba livia*) Fecal Droppings in Western Saudi Arabia

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Abstract

Bird fecal matter is considered a potential source of pathogenic microbes such as yeast species that contaminate the environment. Therefore, it needs to be scrutinized to assess potential environmental health risks. The aim of this study was to investigate the diversity of the yeasts in pigeon fecal droppings, their antifungal susceptibility patterns, and virulence factors. We used culturing techniques to detect the yeasts in pigeon fecal droppings. The isolates were then characterized based on colony morphologies, microscopic examinations, and biochemical reactions. The molecular identification of all yeast isolates was performed by sequencing of the amplified ITS gene. Genes encoding virulence factors *CAP1*, *CAP59*, and *PLB* were also detected. Antifungal susceptibility patterns were examined by the disk diffusion method. A total of 46 yeast-like isolates were recovered, and they belonged to nine different genera, namely, *Cryptococcus*, *Saccharomyces*, *Rhodotorula*, *Candida*, *Meyerozyma*, *Cyberlindnera*, *Rhodosporidium*, *Millerozyma*, and *Lodderomyces*. The prevalence of two genera *Cryptococcus* and *Rhodotorula* was high. None of the yeast isolates exhibited any resistance to the antifungal drugs tested; however, all pathogenic *Cryptococcus* species were positive for virulence determinants like urease activity, growth at 37°C, melanin production, the *PLB* and *CAP* genes. This is the first report on the molecular diversity of yeast species, particularly, *Cryptococcus* species and their virulence attributes in pigeon fecal droppings in Saudi Arabia.

Key words: *Cryptococcus*, pigeon, fecal droppings, antifungal susceptibility, virulence genes, yeast

Introduction

Free-living wild birds are regarded as one of the indicators of a healthy environment. However, they also may be regarded as potential carriers of human-pathogenic viral, bacterial, fungal, and protozoan agents. Free-living rock pigeons (*Columba livia*), are found in large flocks within major cities around the world. They live in close proximity to humans, particularly in public parks, on rooftops, and sometimes close to catering establishments. Numerous reports highlight that pigeon fecal droppings in public areas are a source of bacterial infectious agents, such as *Salmonella*, *Campylobacter*, and *E. coli* O157, and that they may significantly affect public health (Abulreesh et al. 2007; Abulreesh 2014).

Carriage of pathogenic yeast in pigeon feces is a matter of growing interest and has been investigated worldwide, with much focus on the *Cryptococcus* species. Wu et al. (2012) reported the presence of eight different genera of yeast, such as *Cryptococcus*, *Candida*,

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and *Rhodotorula*, in pigeon fecal droppings in Beijing, China. They concluded that pigeon feces are a vector for medically important yeast species. Similarly, in Brazil, Costa et al. (2010) reported the predominance of *Cryptococcus*, *Candida*, and *Rhodotorula* species in pigeon feces. Other reports from Egypt (Mahmoud 1999), the Canary Islands (Rosario et al. 2010), India (Xavier et al. 2013), and Sweden (Mattsson et al. 1999) highlighted the diversity of *Cryptococcus* species found in pigeon fecal droppings and the role of domestic pigeons as potential source of environmental pathogenic yeast. Soltani et al. (2013) even suggested a direct link between pathogenic yeast flora in pigeon feces and human infections.

The genus *Cryptococcus* is a basidiomycete yeast belonging to the *Tremellales* class. The genus *Cryptococcus* consists of two species complexes: *C. neoformans* species complex (comprise of two species: *C. neoformans* and *C. deneofirmans*), and *C. gattii* species complex (comprises of five species: *C. gattii*; *C. tetragattii*; *C. decagattii*; *C. deuterogattii* and *C. bacillisporus*); these species are known for their potential clinical significance. *C. neoformans* species complex is responsible for cryptococcosis in humans, an infection that affects the lungs, the brain, and spinal cord (central nervous system) that usually affect HIV patients and other immunocompromised individuals, while *C. gattii* species complex causes serious infections and immunocompetent as well immunocompromised individuals. These species are commonly found in the excreta of wild birds, particularly pigeons, as well as soil, rotting vegetables, wood, and associated with certain species of plants. Cryptococcosis is not a contagious disease, however, it is acquired from environmental exposure to *Cryptococcus* species. Recent epidemiological data suggest that there are around 220 000 annual cases of cryptococcal meningitis in HIV patients worldwide. Therefore, cryptococcal meningitis may be the leading cause of death among HIV patients (Cogliati 2013; Hagen et al. 2015; Rajasingham et al. 2017; Esher et al. 2018; Magalhães Pinto et al. 2019).

Molecular identification tools such as real-time PCR, multiplex PCR, and RFLP PCR have been successful in detecting and identifying various yeast species in clinical and environmental samples. Furthermore, the identification of yeast-based on fungal ribosomal DNA (rDNA) has become popular as an accurate molecular tool. This tool allows for the detection of 18S and 26S subunits of rDNA that are separated by the internal transcribed spacers ITS1 and ITS2 (Pincus et al. 2007).

The city of Makkah is a major attraction for people around the world visiting for religious and spiritual purposes. Hence, the study of biological contamination of the environment by pigeons may be of great significance from a public health perspective. Very little information exists regarding the diversity and characterization of yeast species found in pigeon fecal droppings in Saudi Arabia, and particularly in Makkah city. The only available report describing the presence of *C. neoformans* in pigeon fecal droppings relied upon the phenotypical characterization of the isolates (Abulreesh et al. 2015). It did not involve the molecular identification of those isolates. The current study is, therefore, the first to report on the molecular diversity of yeast genera and their antifungal susceptibility patterns in pigeon feces within the city of Makkah, western Saudi Arabia. The paper also describes for the first time, the carriage of different genes encoding virulence factors among *Cryptococcus* and other yeast species found in pigeon feces in western Saudi Arabia.

Experimental

Materials and Methods

Sampling. A total of 100 samples of dried pigeon fecal droppings were collected from various locations within the city of Makkah, western Saudi Arabia, between May and November 2018. Each fecal sample was collected in a sterile universal bottle, protected from direct sunlight and transported to the laboratory on ice. All samples were processed within 6 h of collection.

Yeast isolation. Yeast species were isolated from pigeon fecal dropping following the methodology previously described by Abulreesh et al. (2015). First, 10 g of fecal droppings from each location was aseptically transferred to a flask containing 0.9 % saline solution with chloramphenicol (200 μg l^{-1}). Then the mixture was shaken for 20 min and allowed to settle for 30 min. An aliquot of 0.5 ml of each supernatant was streaked onto Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, UK). Plates were incubated at 25°C and were examined daily for ten days to observe the growth of yeast. Individual colonies with the mucous appearance and yeast-like colonial morphology were selected and subcultured on SDA to obtain pure cultures.

Yeast identification. The selected colonies were microscopically examined for typical yeast cell morphology, pseudomycelium (specific for *Candida* spp.), and capsule in Indian ink preparations (in the case of *Cryptococcus* spp.). Biochemical identification for all yeast isolates included carbohydrate and nitrate assimilation (Teodoro et al. 2013), urease reaction on, urea agar base (Christensen's medium) (Oxoid, Basingstoke, UK) (Canteros et al. 1996), production of melanin on esculin agar (Oxoid), and ability to grow at 37°C on SDA (Abulreesh et al. 2015).

Molecular identification. All yeast isolates were identified at a molecular level by the detection of the internal transcribed spacer (ITS) regions. The ITS1-

5.8S-ITS2 fragment was amplified using universal primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) (White et al. 1990). Total DNA was extracted from colonies using the DNeasy Plant Mini kit (QIAGEN, Hilden, Germany), according to the manufacture's procedure. Amplification was performed in 50 µl reaction volume, containing 5.0 µl 10 × PCR Buffer, 4 µl dNTP, 0.5 µl r*Taq*, 1.0 µl ITS1 and ITS4, 3.0 µl genomic DNA, and 35.5 µl distilled water. The PCR protocol comprised of initial denaturation at 94°C for 5 min, followed by 35 cycles of 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) and sequenced in both directions using the amplification primers on an ABI3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). BLASTN (http://blast.ncbi.nlm.nih.gov) was employed to confirm the identified strains (Wu et al. 2012).

Detection of virulence-encoding genes. The molecular detection of virulence-encoding genes, such as the capsular genes (*CAP1* and *CAP59*) specific for *Cryptococcus* spp. and the phospholipase gene (*PLB1*) was performed on yeast isolates previously identified by ITS sequencing. *CAP1* is a 700 bp long gene on chromosome IV and is a part of the *MAT* locus that encodes a capsule-synthesis associated protein. Primers used for *CAP1* were, 5'-CGTTCGCGATAGAGAGAGGA-3' (forward) and 5'-CCTTACCTTCACAGTCGCCC-3' (reverse). *CAP59* is a 502 bp long gene on chromosome I and encodes for a capsule-synthesis associated protein. Primers used for *CAP59* were, 5'-CTCTACGTCGAGCAAGTCAAG-3' (forward) and 5'-TCCGCTGCACAAGTGATACCC-3' (reverse). *PLB1* is an 853 bp long gene, present on chromosome XII, encoding a phospholipase B probably involved in cell invasion. Primers used for *PLB1* were, 5'-CTTCAGGCGGAGAGAGGTTT-3' (forward) and 5'-GATTTGGCGTTGGTTTCAGT-3' (reverse) (Chowdhary et al. 2011). Each PCR reaction mixture comprised of 2.0 µl (~ 1ng) of the template DNA, 8.0 µl of GoTaq (*Taq* DNA polymerase + $MgCl_2$ at a final concentration of 1.5 mM, supplied by Promega USA), 0.2 µM of each primer, and 5.8 µl of sterile distilled water to make up a total volume of 16 µl. The thermal cycling protocol included an initial denaturing step at 95°C for 4 min. This was followed by 40 cycles of denaturation at 95°C for 1 min, primer-specific annealing temperature for 1 min, and extension at 72°C for 1 min. The final step was the primer extension at 72°C for 7 min. Primer-specific annealing temperatures for amplifying the nine gene fragments were: 59.2°C for *CAP1*, 55°C for *CAP59*, and 56°C for *PLB1* (Chowdhary et al. 2011).

Antifungal susceptibility testing. To determine the antifungal susceptibility patterns, the agar disk diffusion method was employed as described by CLSI (2008). Four antifungal agents were used to examine the susceptibility patterns of the yeast species recovered from pigeon feces: Ciclopirox-Olamine (50 µg); Clotrimazole (10 µg); Nystatin (100 IU) and Fluconazole (25 µg), disks were purchased from Liofilchem Inc. (Waltham, USA). Fresh culture of each of the identified species was inoculated using swabs on the surface of Muller Hinton agar (Oxoid), plates supplemented with 2.0% glucose. Plates were incubated for 36 h at the optimum temperature for each fungal species. The sensitivity of the species against the antifungal compounds was determined by measuring the diameter of inhibition zones. A diameter of ≥ 15 mm for nystatin and ≥ 20 mm for the three antifungal agents was considered as susceptible. Susceptibility breakpoints of antifungal drugs used in our study are not species related (CLSI 2008). *C. albicans* ATCC 90028 was used as control.

Results

Yeast diversity in pigeon feces. A total of 46 presumptive yeast isolates representing nine different genera, were recovered from pigeon fecal samples collected within the city of Makkah. Table I consolidates the phenotypic characterization and molecular identification of each isolate. *Cryptococcus* spp. accounted for 41.3% of all the isolates (19 isolates). There were four different species belonging to the genus *Cryptococcus*; *C. neoformans* (11 isolates); *C. albidus* (5 isolates), *C. gattii* (2 isolates), and *C. liquefaciens* (1 isolate) (Fig. 1 and 2). Surprisingly, *Candida* spp. were almost absent, with only one isolate (2.1%) identified to be *C. glabrata*, however, other *Candida*-related genera such as *Meyerozyma guilliermondii* (8.7%) were present. *Saccharomyces cerevisiae* made up 10.9% of the isolates recovered from pigeon feces (Fig. 1), *Lodderomyces elongisporus*, *Millerozyma farinosa*, and *Cyberlindnera fabianii* were also present at an abundance of 4.34%, 6.5%, and 6.5%, respectively (Fig. 1). Two species belonging to *Rhodotorula*, *R. glutinis* (4.34%) and *R. mucilaginosa* (10.9 %) as well as *Rhodosporidium plaudigenum* (4.34 % were identified in the pigeon fecal samples (Table I, Fig. 1).

Detection of virulence factors. The virulence factors were detected using (i) conventional methods, such as urease activity, and melanin production, and (ii) molecular methods probing for capsular (*CAP1* and *CAP59*) and phospholipase (*PLB1*) genes.

Table I exhibits the urease activity and melanin production of all isolates. Urease activity was observed in all the four isolated *Cryptococcus* species. *R. glutinis* and *R. plaudigenum* were the only non-*Cryptococcus* species that showed positive activity for urease. Melanin production was only observed in all *Cryptococcus* species.

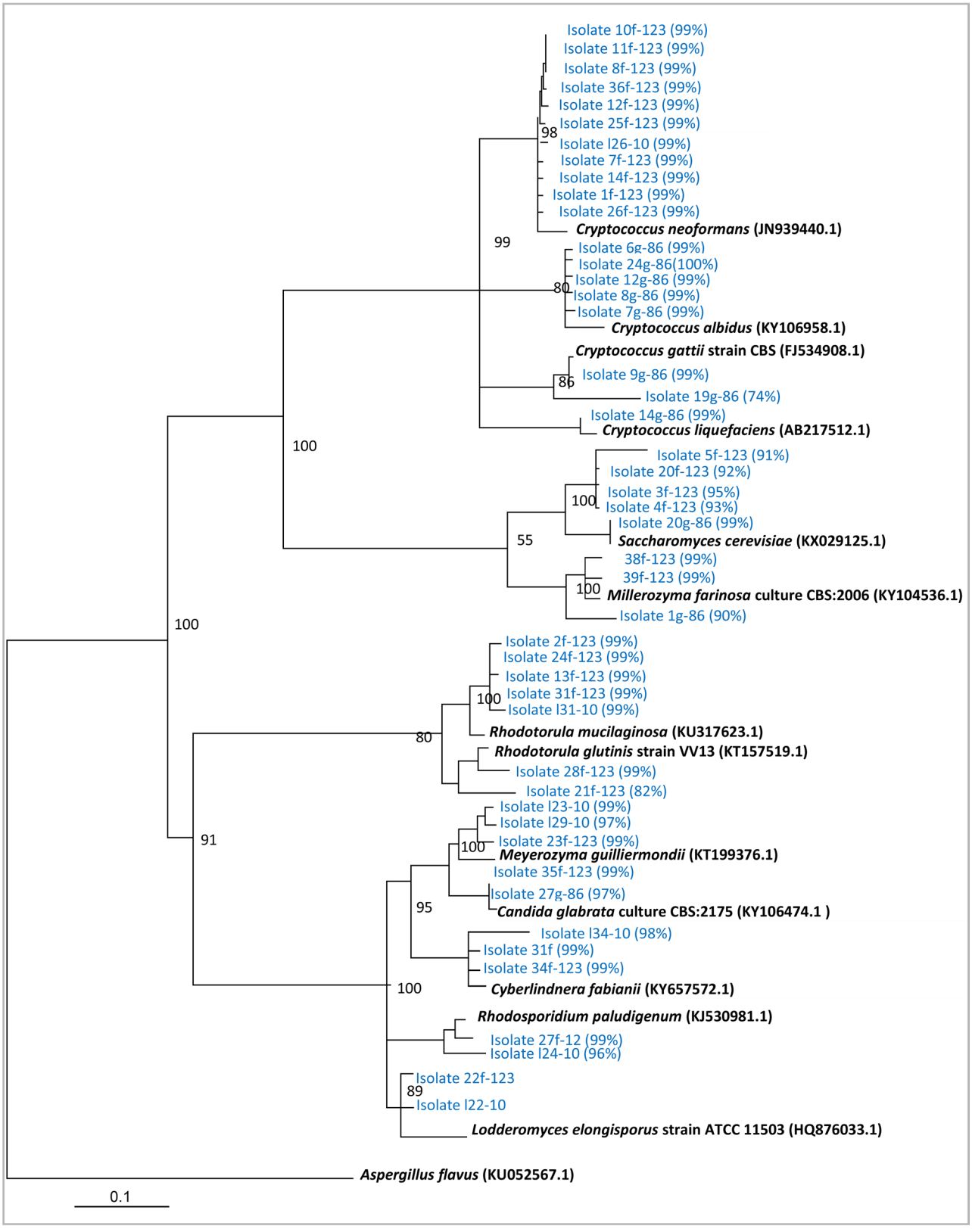

Fig. 1. Neighbor-joining tree showing the estimated phylogenetic relationship of the all fungi and yeasts strains (shown in blue) and other closely related strains. Bootstrap values out of 100 are given at the nodes based on the sequence of ITS region (amplify the ITS1-5.8S-ITS2 fragment). *Aspergillus flavus* (KU052567.1) was used as out group.

Table II shows the presence and absence of the *CAP1, CAP59* and *PLB1* genes for all isolates. The *CAP1*gene was detected in six of 11 *Cryptococcus neoformans* isolates, while the *CAP59* gene was detected in the other five isolates. Two isolates of *C. gattii* also possessed the *CAP59* gene (Fig. 3). The *PLB1* gene

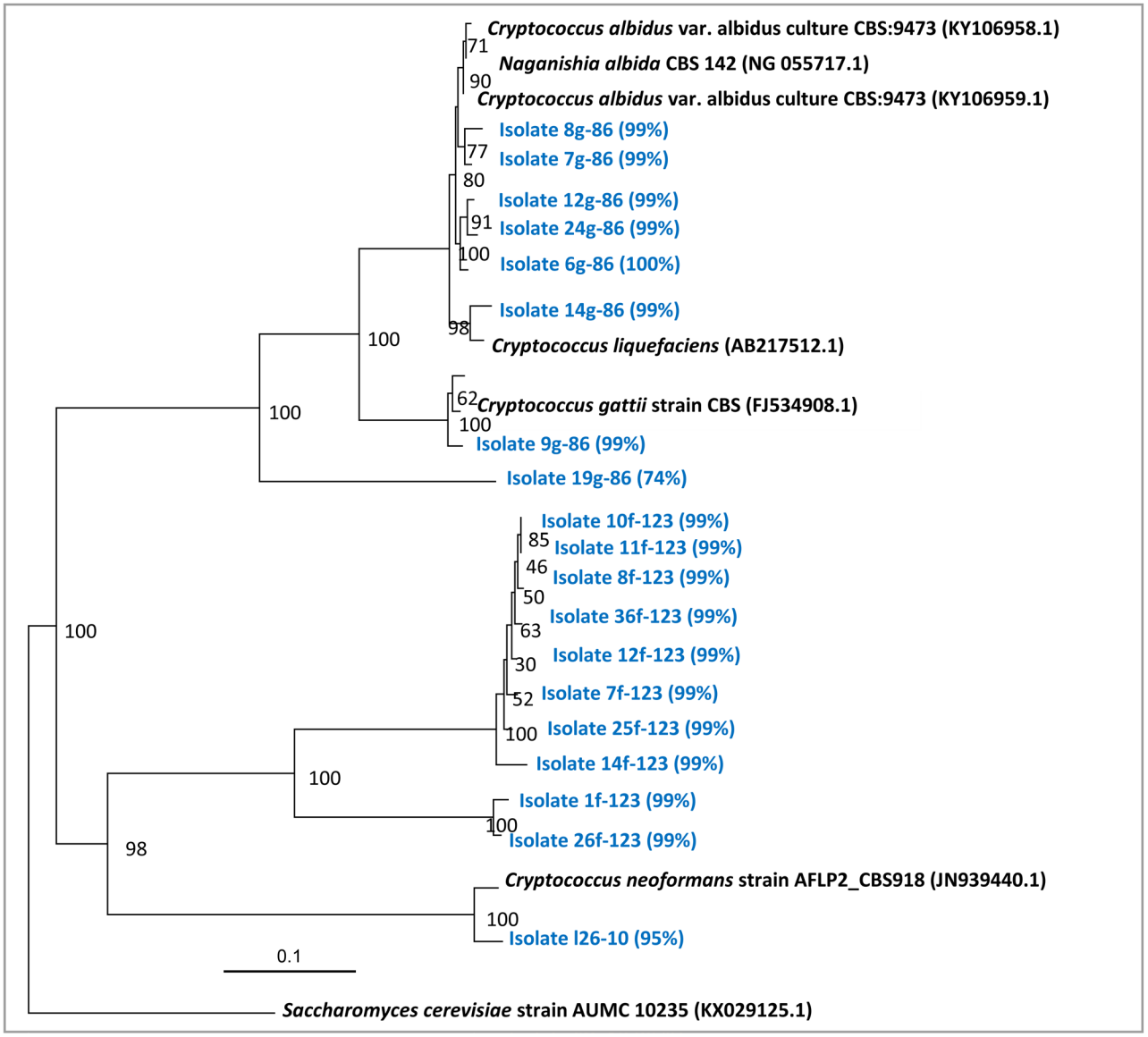

Fig. 2. Neighbor-joining tree showing the estimated phylogenetic relationship of the *Cryptococcus* strains (shown in blue) and other closely related strains. Bootstrap values out of 100 are given at the nodes, based on the sequence of ITS region (amplify the ITS1-5.8S-ITS2 fragment). *Saccharomyces cerevisiae* strain AUMC 10235 (KX029125.1) as out group

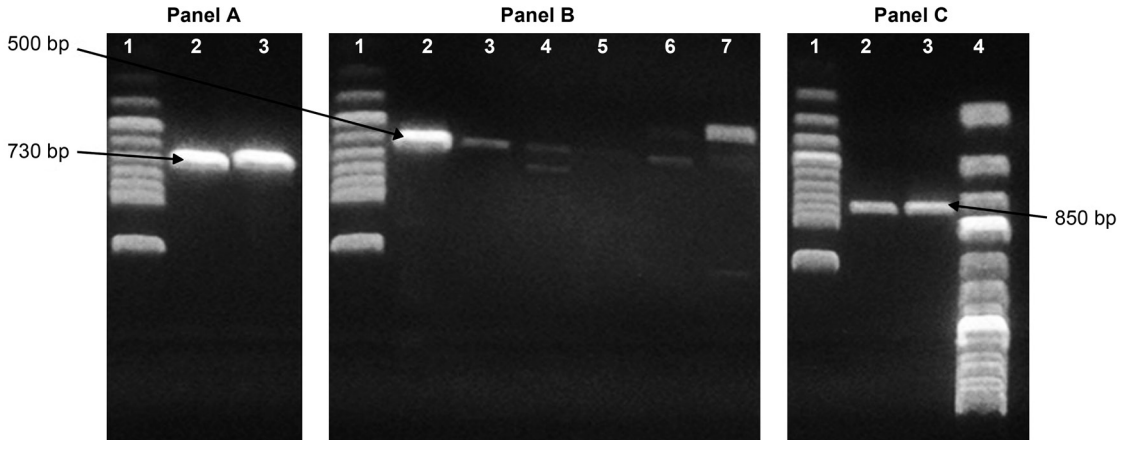

Fig. 3. Ethidium bromide stained agarose gel electrophoresis resolving the PCR Screening amplification fragments for the presence of CAP1 panel A lanes: 2 (Y33), 3 (Y38) (~730 bp), CAP59 panel B lanes 2 (Y1), 3 (Y3), 4 (Y21), 5 (Y5), 6 (Y20) and 7 (Y9) (~520 bp) and PLB1 Panel C lanes: 2 (Y44) and 3 (Y46) (~853 bp). Lane 1: 100 bp marker and lane 4 panel C: 1 Kbp ladder. Y1, Y5,Y9, Y20, Y33, Y38, Y46 = *Cryptococcus neoformans* Y44 = *Saccharomyces cerevisiae* Y3, Y21 = *Cryptococcus gattii*

Table I
Molecular identification and phenotypical traits of yeast isolates in pigeon feces.

Yeast isolates ITS ID	Number of isolates	Phenotypic traits								
		Nit*	Melanin	Urease	Pseudomy*	Capsule	Glu*†	Lact*†	Sucr*†	37°C‡
Cryptococcus neoformans	11	–	+	+	–	+	+	–	+	+
Cryptococcus albidus	5	+	+	+	–	+	+	+	+	+
Cryptococcus gattii	2	–	+	+	–	+	+	–	+	+
Cryptococcus liquefaciens	1	+	+	+	–	+	+	+	+	–
Saccharomyces cerevisiae	5	–	–	–	–	–	+	–	+	–
Millerozyma farinosa	3	–	–	–	–	–	+	+	–	+
Rhodotorula mucilaginosa	5	–	–	+	–	+	+	–	+	–
Rhodotorula glutinis	2	–	–	+	–	+	–	–	–	+
Meyerozyma guilliermondii	4	–	–	–	+	–	+	–	+	–
Candida glabrata	1	–	–	–	–	–	+	–	–	+
Cyberlindnera fabianii	3	+	–	–	–	–	+	–	+	+
Rhodosporidium paludigenum	2	+	–	+	–	–	+	–	+	–
Lodderomyces elongisporus	2	–	–	–	+	–	+	+	–	+

Nit* – nitrate reduction, Glu* – Glucose, Lact* – Lactose, Sucr* – Sucrose, Pseudomy* – Pseudomycelium,
Glu†, Lact†, Sucr† – assimilation
‡ – ability to grow at 37°C

Table II
Detection of virulence factors-encoding genes in yeast species isolated from pigeon feces.

Yeast isolates ITS ID	Total number of isolates	Virulence factors-encoding genes		
		CAP1	*CAP59*	*PLB*
Cryptococcus neoformans	11	6	5	11
Cryptococcus albidus	5	–	–	5
Cryptococcus gattii	2	–	2	2
Cryptococcus liquefaciens	1	–	–	–
Saccharomyces cerevisiae	5	ND	ND	5
Millerozyma farinosa	3	ND	ND	–
Rhodotorula mucilaginosa	5	ND	ND	–
Rhodotorula glutinis	2	ND	ND	–
Meyerozyma guilliermondii	4	ND	ND	–
Candida glabrata	1	ND	ND	1
Cyberlindnera fabianii	3	ND	ND	–
Rhodosporidium paludigenum	2	ND	ND	–
Lodderomyces elongisporus	2	ND	ND	–

ND – not determined

was detected in three *Cryptococcus* species, *C. neoformans*; *C. albidus*, and *C. gattii* but not in *C. liquefaciens*. *C. glabrata* and *S. cerevisiae* were also positive for the *PLB1* gene (Fig. 3).

Antifungal susceptibility patterns. None of the 46 yeast isolates showed resistance to any of the antifungal drugs, i.e., Ciclopirox-Olamine; Clotrimazole; Fluconazole and Nystatin used in this study (Table III).

Discussion

The presence of yeast species, especially the pathogenic ones, in pigeon fecal droppings and the role of pigeons in the dissemination and epidemiology of these pathogenic species have been investigated worldwide. However, in Saudi Arabia, there is a dearth of information regarding the environmental diversity of yeast,

Table III
Antifungal susceptibility patterns of yeast species isolated from pigeon feces (diameter zone, mm).

Yeast species	Number of isolates	Antifungal agents (A)‡			
		Ciclopirox-Olamine	Clotrimazole	Fluconazole	Nystatin
Cryptococcus neoformans	11	29 (S)	41 (S)	51 (S)	22 (S)
Cryptococcus albidus	5	33 (S)	46 (S)	51 (S)	22 (S)
Cryptococcus gattii	2	31 (S)	46 (S)	51 (S)	28 (S)
Cryptococcus liquefaciens	1	28 (S)	50 (S)	56 (S)	25 (S)
Saccharomyces cerevisiae	5	54 (S)	55 (S)	60 (S)	35 (S)
Millerozyma farinosa	3	41 (S)	45.3 (S)	57 (S)	25 (S)
Rhodotorula mucilaginosa	5	42 (S)	51 (S)	60 (S)	35 (S)
Rhodotorula glutinis	2	58 (S)	60 (S)	56 (S)	40 (S)
Meyerozyma guilliermondii	4	56 (S)	57.3 (S)	53 (S)	48 (S)
Candida glabrata	1	33 (S)	38 (S)	48 (S)	22 (S)
Cyberlindnera fabianii	3	47 (S)	45 (S)	65 (S)	51 (S)
Rhodosporidium paludigenum	2	53 (S)	50 (S)	60 (S)	44 (S)
Lodderomyces elongisporus	2	51 (S)	51 (S)	58 (S)	35 (S)
Candida albicans ATCC 90028	1	27 (S)	30 (S)	39 (S)	22 (S)

S – Susceptible
A‡ – Average inhibition zone reading of all isolates

including the pathogenic species. Abulreesh et al. (2015) were the first to report about *C. neoformans* in pigeon fecal droppings in Saudi Arabia. Their report was based on phenotypical identification of the presumptive isolates, and no molecular identification was performed to confirm the results. The aim of this work was to further investigate the diversity of *Cryptococcus* and other yeast species in pigeon excreta, in addition to their virulence factors and antifungal susceptibility patterns.

Diversity of yeast species in pigeon feces. In this study, nine different genera of yeast were found in pigeon fecal droppings, in comparison to other studies conducted elsewhere, the current study has identified the most diverse yeast species ever reported. For example, Wu et al. (2012) reported six different yeast species in pigeon feces from various locations in Beijing, China. In our study, we reported *C. neoformans*, *C. albidus*, and *C. gattii*, with *C. neoformans* being the most prevalent than other species. These species have frequently been associated with pigeon fecal droppings in Brazil (Costa et al. 2010), China (Wu et al. 2012), the Canary Islands (Rosario et al. 2010), Iran (Hashemi et al. 2014), Thailand (Tangwattanachuleeporn et al. 2013), India (Xavier et al. 2013), Mexico (Canónico-González et al. 2013), Korea (Chae et al. 2012), and countries in the Middle East and North Africa region (Mahmoud 1999; Mseddi et al. 2011; Abbass et al. 2017). One of our isolates is *C. liquefaciens*, which has not been reported in pigeon excreta to the best of our knowledge. We are the first to report its presence in pigeon excreta in the Middle East and Asia. In General, *Cryptococcus* spp. thrive in pigeon fecal droppings due to the high content of urea and organic matter (Costa et al. 2010; Abulreesh et al. 2015).

Similar to the previous studies, we found that *S. cerevisiae* is less common than other saprophytic yeast species in pigeon feces (Wu et al. 2012; Rosario Medina et al. 2017). Although *S. cerevisiae* has no veterinary significance, its presence in bird excreta may be due to the physico-chemical nature of the droppings that provide a rich environment for yeasts to grow (Cafarchia et al. 2008).

Candida species, especially *C. albicans* have also been frequently found in pigeon excreta (Wu et al. 2012; Rosario Medina et al. 2017). However, *C. albicans* was not detected in our study, instead, *C. glabrata*, was the only *Candida* species encountered. Other *Candida*-related genera, *M. guilliermondii* (formerly *Candida guilliermondii*), and *Cyberlindnera fabianii* (formerly *Candida fabianii*), were also present in this study, the former has been isolated in pigeon feces in Spain (Rosario Medina et al. 2017), while the latter has been very rarely detected in pigeon excreta.

We detected two other genera in our study, *M. farinosa* (formerly *Pichia farinosa*), and *Rhodosporidium paludigenum*. Both have not been, detected in pigeon or other bird excreta worldwide. On the other hand, *R. mucilaginosa* and *R. glutinis* are very commonly found in pigeon fecal droppings (Wu et al. 2012; Marenzoni et al. 2016; Abbass et al. 2017). However, *Lodderomyces elongisporus* has seldom been reported in pigeon fecal droppings (Wu et al. 2012). It is worth noting that due to the method of sampling we adopted in the current study,

environmental contamination of fecal samples (e.g. from soil) cannot be ruled out and may play, in part, role in the diverse yeast genera reported in our study.

Virulence factors of yeast species isolated from pigeon excreta. Virulence factors play a vital role in pathogenesis. Various *Cryptococcus* species, like *C. neoformans*, *C. gattii*, and *C. albidus* possess an arsenal of such molecules. These factors enable them to successfully invade hosts, to resist defense mechanisms of their immune system and to cause infection, especially in the immunocompromised individuals. The prominent capsule of *Cryptococcus* species is an important virulence factor. All the *Cryptococcus* species isolated in this study displayed capsules in Indian ink preparations, as observed under the microscope. On a molecular level, the *CAP1* gene was detected in 54.55% of *C. neoformans* isolates, whereas, the *CAP59* gene was detected in the rest of the isolates. The *C. gattii* exhibited only the *CAP59* gene. The formation of the capsule by the *Cryptococcus* species was induced by many environmental conditions that include pH, CO_2 levels and iron deprivation (Alspaugh 2015). The polysaccharide capsule helps *Cryptococcus* species to proliferate within the phagocytic cells and to inhibit host any immune response. Once it has invaded the host cell, and colonized the vacuole, *Cryptococcus* will survive by the aid of the capsule and replicate despite the acidic nature of the vacuole (Srikanta et al. 2014). Both of the *CAP1* and *CAP59* genes are specific to *C. neoformans* and *C. gattii*, this explains the absence of these genes in other encapsulated isolates such as *C. liquefaciens*, *C. glabrata*, and *Rhodotorula* species.

Phospholipase is another virulence factor that helps the pathogenesis of *Cryptococcus* and other pathogenic yeast species. Phospholipase activity can alter the microenvironment of infection and can facilitate *Cryptococcus* species to survive better within the host cells (Alspaugh 2015). All *Cryptococcus* species detected in this study, except for *C. liquefaciens*, were positive for the *PLB1* gene, it was also detected in *C. glabrata*. The presence of the *PLB1* gene was also noted in *S. cerevisiae* isolates. The ability of phospholipase to hydrolyze phospholipids and to produce several bioactive compounds has given *S. cerevisiae* its industrial potential.

Melanin is a known protective determinant for *Cryptococcus* against environmental stressors and, hence, considered a virulence factor (Alspaugh 2015). Similarly, urease activity has been associated with pathogenesis, in pathogenic yeasts such as the *Cryptococcus* species. The ammonia produced by urease activity damages the host cell endothelium; thus, the yeast to transmigrate toward the central nervous system (Feder et al. 2015). It is suggested that capsules, melanin production, and high-temperature growth (at 37°C) are key virulence determinants for pathogenic and opportunistic yeasts (Boral et al. 2018). All these characteristics were observed for the isolates of *Cryptococcus* species in this study.

In general, several environmental yeast species possess the similar virulence factors as their pathogenic clinical counterparts (Magalhães Pinto et al. 2019). Our study reports this notion and highlights the presence of pathogenic yeast in the fecal droppings of free-living pigeons, suggesting the pathogenic potential of these environmental species of yeast.

Antifungal susceptibility. None of the 46 yeast isolates representing the nine different genera reported here, was resistant to the antifungal drugs tested. These results correlate with previously reported studies, particularly for *Cryptococcus* species, that it is rare for environmental (pigeon-derived) *C. neoformans* and *C. gattii* to exhibit resistance to antifungal drugs (Costa et al. 2010; Souza et al. 2010; Tangwattanachuleeporn et al. 2013; Teodoro et al. 2013). The clinical isolates of *Cryptococcus* species exhibit similar trends of low resistance to antifungal drugs (Souza et al. 2010; Govender et al. 2011). Environmental and clinical samples of *C. glabrata* have previously exhibited susceptibility to some of the antifungal drugs used in this study (Nenoff et al. 2011; Lotfalikhani et al. 2018; Miranda-Cadena et al. 2018). The lack of antifungal resistance was also observed in clinical *R. mucilaginosa* isolates (Razzaq Abed and Mohammed Hussein 2017). Resistance to fluconazole appears to be common in clinical isolates of *R. mucilaginosa*, this perhaps due to the fact that most of the patients are administered with fluconazole when fungemia is diagnosed (Wirth and Goldani 2012). The resistance mechanism of *Rhodotorula* to fluconazole is not known, thus, the observation of repeated resistance may suggest intrinsic resistance in some isolates (Duggal et al. 2011). So far, no available reports have described antifungal susceptibility patterns of environmental *Rhodototrula* species, therefore, whether environmental isolates exhibit similar resistance patterns, particularly to fluconazole, remains to be elucidated.

There is a lack of scientific evidence on the antifungal susceptibility patterns of both environmentally and clinically derived isolates of *M. farinose*, *S. cerevisiae*, *R. glutinis*, *M. guilliermondii*, *C. fabianii*, *R. paludigenum*, and *L. elongisporus*. Hence, we are unable to compare and discuss our results for these yeast species.

The lack of resistance observed in these environmental isolates of yeasts, particularly pathogenic species, may be explained by the role of various environmental factors that are not fully understood. Possibly there might be unique ecological niches in the environment where environmental yeast species can acquire drug resistance, or there might be a pattern of spread of drug resistance among environmental yeast species through geo-climatic factors, such as wind activity or

global warming that yet to be explored. Bird migration has been playing an important role in the spread of multidrug resistance in bacteria; it is not clear whether it plays a similar role in yeast drug resistance (Kontoyiannis 2017). Furthermore, it was hypothesized that environmental yeast could acquire resistance to antifungal drugs in the presence of industrial waste of pollutants that could promote altered expression of genes that may occasionally occur in pathways related to resistance (Milanezi et al. 2019). It is also possible that the disk diffusion method may have influenced the susceptibility results. It is highly likely that the use of dilution method or MIC test strips would provide more accurate susceptibility results in comparison to the disk diffusion method, i.e. some of the isolates might have exhibited resistance if tested by the dilution method or MIC strips.

Public health significance of this study. Earlier studies have implicated that free-living pigeons spread pathogens in the environment and have established a direct link between pigeon droppings and human infections (Haag-Wackernagel and Moch 2004). In this study, we observed three different species of pathogenic *Cryptococcus* in pigeon fecal droppings in western Saudi Arabia: *C. neoformans*, *C. gattii*, and *C. albidus*. Together they make up around 39% of all yeast species found in the excreta. Different *Cryptococcus* species have been reported to cause human infections, *C. neoformans* and *C. gattii* for cryptococcosis (Cogliati 2013), *C. albidus* for fungemia (Cleveland et al. 2013), and respiratory infections (Burnik et al. 2007). In Saudi Arabia, there is only one incidence of a clinical case involving *C. neoformans*. The pathogen caused abscess and osteomyelitis in an immunocompetent individual (Al-Tawfiq et al. 2007). However, this lack of reported incidents may not truly reflect low occurrence of clinical cases in the region. In contrast, we suggest that there may have been relatively few clinical investigations of medically important fungi.

Other yeast species found in pigeon feces in this study are also implicated in human infections. *C. glabrata* has been implicated in various diseases in humans including both superficial and systematic infections, such as brain abscess (Zhu et al. 2018), vertebral column (spondylodiscitis) infection (Gagliano et al. 2018), joint infection (Koutserimpas et al. 2018), and cutaneous granuloma (Fan et al. 2018).

R. mucilaginosa is an environmental yeast that has emerged as a causative agent of serious and even fatal opportunistic infections, including fungemia (Kitazawa et al. 2018) and meningitis (Miceli et al. 2011) particularly in immunocompromised patients, and immunocompetent individuals. Other species identified in our study that have been implicated in severe or fatal human infections include: *C. fabianii* (Hof et al. 2017), *L. elongisporus* (Hatanaka et al. 2016), *M. guilliermondi* (Cebeci et al. 2017), and the emerging invasive infections of *S. cerevisiae* (Popiel et al. 2015).

The most commonly transmitted pathogens via pigeons continue to be *Chlamydophila psittaci* and *C. neoformans* (Haag-Wackernagel and Moch 2004). Considering the relationship between environmental *C. neoformans* strains and human infection, Delgado et al. (2005) concluded that cryptococcosis could be acquired from the environmental strains in both urban and rural areas. Liaw et al. (2010) drew similar conclusions, finding strong similarities between clinical and environmental strains of *C. neoformans* and suggesting that patients might acquire yeast infection from the environment. Overall, the results of our study demonstrate that pigeon fecal droppings carried a number of pathogenic yeast species as well as emerging opportunistic yeast genera.

In Makkah city, western Saudi Arabia, massive numbers of pigeons inhabit the city. They flock in public parks, rooftops, and in close proximity to catering areas, making it almost unavoidable for humans to come in contact with their droppings. Pigeon excreta can be noted almost everywhere in the city. Additionally, dust from dried pigeon fecal droppings may contain different cryptococcal species and other opportunistic yeast. Despite the massive cleaning efforts of public spaces, dissemination of pathogenic yeast by air is inevitable, increasing the chances of acquiring infection through the respiratory system.

Risk assessment of environmental *Cryptococcus* species. *C. neoformans* and *C. gattii* are pathogenic yeasts that rarely cause infections in healthy individuals. However, immunocompromised patients, such as those who underwent organ transplant, those under medications that weaken the immune system (e.g. corticosteroid or rheumatic arthritis medications), or people with an advanced stage of HIV infection are at a high risk of getting *C. neoformans* infection. In addition, elderly individuals, over 50 years old with lung health issues may be at risk of *C. gattii* infection (Cogliati 2013). *Cryptococcus* infection is not contagious and there is low risk for healthy people to be infected when in contact with an infected individual (Delgado et al. 2005). However, *Cryptococcus* can infect healthy people when they inhale the dust containing the pathogens. This is common in the environment, in areas where pigeon fecal droppings exist in abundance. Every individual may inhale the yeast dust, yet they may not develop any symptoms immediately. *Cryptococcus* can stay hidden within the body and cause infection later, when the immune system is too weak to fight it (Esher et al. 2018). Currently, many countries around the world do not consider the detection of *Cryptococcus* in clinical routine work, particularly in meningitis cases. To mitigate the risk of *Cryptococcus* infections, it is necessary

to perform extensive surveillance of the environmental distribution of the pathogens and laboratory detection of *Cryptococcus* infections in clinical specimens. Early detection of *Cryptococcus* infection in individuals may help to treat them promptly and to reduce the mortality rate of infected people.

Conclusions

Pigeon fecal droppings in western Saudi Arabia were found to harbor a wide range of pathogenic and opportunistic yeast species. Although none of them were resistant to the common antifungal drugs, all pathogenic species and some of the opportunistic species did carry different virulence factors. Our study confirms that pigeon fecal droppings provide a rich environment for the growth of saprophytic yeasts, particularly *Cryptococcus* species. Additionally, we demonstrate that pigeons may act as reservoirs and carriers not only for pathogenic yeast (e.g. *Cryptococcus* and *Candida*), but also for opportunistic yeast species (e.g. *R. mucilaginosa*). This is the first report on the diversity and virulence factors of yeast species in pigeon fecal droppings in Saudi Arabia. Further investigations are required to understand the pathogenicity of these isolates in humans and animals using suitable experimental models.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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