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Phylogenetic analysis of pathogenic *Candida spp.* in domestic pigeons

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Abstract

The current study was conducted to survey the prevalence of pigeon candidiasis in diseased pigeons suspected to candidiasis by isolation, microscopic examination, and polymerase chain reaction (PCR) method and to characterize *Candida spp.* phylogenetically. For this purpose, samples were obtained from 100 suspected pigeons from September 2018 to February 2019 in Ahvaz, Iran. Cloacal and oropharyngeal swab samples were collected from each diseased pigeon with diarrhea resistant to the antibiotics, crop stasis, white diphtheritic membrane in the mouth, regurgitation, and vomiting. Sabouraud dextrose agar was used as a culture medium. Selected colonies were stained with lactophenol cotton blue stain. In the culture and direct microscopic observation, 19.00% of birds were suspected to candidiasis. Twenty-two isolates were identified. All 22 isolates were confirmed as *Candida spp.* By PCR method. The PCR test confirmed the presence of *Candida spp.* in 19.00% of pigeons. Based on the sequencing results of some PCR products, the isolates belonged to *Candida albicans* and *Candida glabrata.* The results revealed a 99.78% accordance when compared with other sequences of *C. albicans* which were formerly deposited in GenBank® from Colombia, Indonesia, China, and Sudan. The results revealed a 99.54% accordance when compared with other sequences of *C. glabrata* which were formerly deposited in GenBank® from the Netherlands and Spain. The symptoms such as diarrhea resistant to antibiotics, crop stasis, white diphtheritic membrane in the mouth, regurgitation, and vomiting were the most prevalent clinical symptoms in positive pigeons.

Introduction

Pigeons and other birds can carry pathogens such as viruses, bacteria, protozoa, and fungi that can be dangerous to public health. For example, pigeons can carry *Cryptococcus species*,[1-3] especially *Cryptococcus neoformans*.[4] Nevertheless, in addition to *Cryptococcus*, other yeasts can be dangerous to public health. Currently diseases caused by *Candida* species have also been considered.[1] Thus, it is important to study the yeasts in pigeons. Candidiasis is an opportunistic fungal disease in poultry that has also been reported to cause disease in wild birds that grow in captivity.[5] *Candida albicans*, the main cause of candidiasis, is a common environmental organism that is a normal inhabitant of the avian crop. Birds such as Passeriformes, Galliformes, Anseriformes, Psittaciformes, and Columbiformes may develop candidiasis.[6] Young birds with crop stasis are more susceptible to the disease.[7] Candidiasis is almost always secondary to other diseases due to suppression of the immune system or after long-term antibiotic treatment. The long-term antibiotic therapy destroys the bacterial flora of the gastrointestinal tract resulting in creating a sensitive environment for yeast colonization in the gastrointestinal tract. One of the factors that make birds susceptible to yeast infection is the age of the bird as young birds with immature immune systems are more sensitive.[7]

In pigeons, severe pressure from flying or racing may cause candidiasis.[8] Contamination of drinking and eating utensils and low level of hygiene in the nest, as well as nutritional deficiencies, especially vitamin A deficiency, are predisposing factors for candidiasis.[8] The digestive system of birds is often affected by yeast infections. The most-reported sign is crop stasis. Anorexia, regurgitation, and subsequent weight loss may also be seen. A specific candida lesion is prominent white plaques that may form on the oropharyngeal mucosa, which may be accompanied by a foul odor.[7] Macroscopic lesions should be distinguished from trichomoniasis, hypovitaminosis A, internal papillomavirus, and pox virus.[9] Appropriate

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handling of candidiasis involves rapid treatment of it. So, any lag in antifungal treatment may result in a severe illness or even death. As a result, fast, accurate, affordable, and timely diagnosis is important. Considering that many pigeons in the Ahvaz region are referred to the veterinary hospital with the mentioned symptoms, and due to the importance of differential and rapid diagnosis to apply appropriate treatment, this study was performed to diagnose and characterize *Candida spp.* phylogenetically.

Materials and Methods

Sampling. The current study was conducted from September 2018 to February 2019 in Ahvaz, Khuzestan province, Iran. One hundred of diseased pigeons (*Columba livia domestica*) which were introduced to the Department of Avian Medicine, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran, were examined. Most of the sick pigeons had nonspecific signs such as losing weight, anorexia, and lethargy. In the history of most diseased pigeons, there had been prolonged use of antibiotics, inadequate hygiene of the birds' environment, and simultaneous suppressive conditions of the immune system (such as malnutrition). Based on the history presented by the owners, and the clinical examination, these birds had clinical symptoms like diarrhea, crop stasis, regurgitation, vomiting, and there were white diphtheritic membranes in their mouths. Cloacal and oropharyngeal swab samples were taken from each suspicious bird. For cloacal sampling, the area was cleaned with disinfectant iodine solution (10.00%) and the swab was inserted into the cloaca and then rotated. For oropharyngeal sampling, the pigeon's beak was slowly opened, and a swab was inserted into the oropharynx. From birds with diarrhea, cloacal swabs were obtained. From birds with crop stasis, regurgitation, vomiting, and the existence of white diphtheritic membranes in the mouth, oropharyngeal swabs were obtained. From birds with diarrhea as well as crop stasis, the existence of white diphtheritic membranes in the mouth, regurgitation, and vomiting, both oropharyngeal and cloacal swabs were obtained. All specimens were kept in sterile vials separately at 4.00 ˚C and were immediately carried to the laboratory for further processing. This study was approved by the Shahid Chamran University of Ahvaz Ethical Commission for Animal Experiments under verification number EE/97.24.3.70424/scu.ac.ir.

Culture and isolation. Sabouraud dextrose agar (SDA; Biolife, Milan, Italy) including 50.00 mg L^{-1} chloramphenicol (Sigma, St. Louis, USA) was used for culturing of all samples (cloacal and oropharyngeal swabs). To evaluate the growth of the yeast, the plates were incubated at 37.00 ˚C for 48 hr. In the current study, based on the visual assessment by researchers of the growth density, samples comprising pure colonies which were grown in zones 3 and 4 of the plate were evaluated severely infected and purified for subsequent evaluation (Fig. 1A). Single colonies with smooth and pasty mucoid shapes were chosen and sub-cultured on SDA and pure cultures were used for molecular detection by PCR method. The samples that grew only in the first and second zones of the plate were evaluated as yeasts that were purely microbial flora (opportunistic *Candida spp.)* and were not subsequently evaluated because this study aimed to evaluate only patient specimens suspected of candidiasis and pathogenic *Candida spp*.

Microscopic examination. To evaluate the presence and number of germinated yeasts, the selected colonies were stained with lactophenol cotton blue (Merck, Darmstadt, Germany) stain and the direct examination was performed under a light microscope (Olympus, Tokyo, Japan) at 400 × magnification (Fig. 1B).

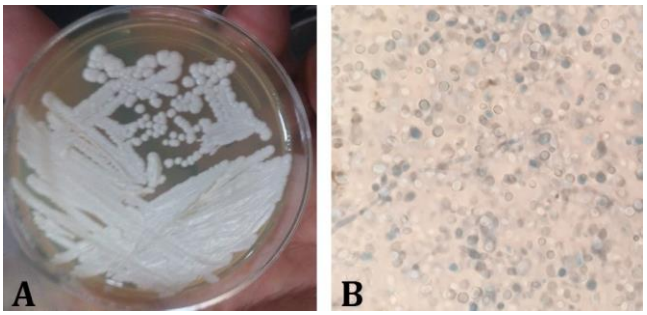

Fig. 1. A) Growth of *Candida spp.* yeast on SDA medium. **B)** The selected colonies were stained with lactophenol cotton blue stain and *Candida spp,* produced true germ tubes under 400×.

DNA extraction and PCR. Two colonies that had a similar morphology to this yeast were removed from the sheer culture of each isolate by flame and were poured into 100 µL of distilled water and vortexed.[10] In a thermoblock (Kiagene, Tehran, Iran), this suspension was boiled for 10 min at 100 ˚C and then centrifuged at 3,000 rpm for 5 min and the supernatant was poured into a new microtube, and it was used as a template in the PCR. The PCR was performed using the forward primer DH (F-ATGGGTGGTCAACATACTC), and reverse primer 1558 (R-TACATCTATGTCTACCACC) which were planned to amplify cytochrome P-450 lanosterol-a-demethylase (L1A1) gene and characterized by Burgener-Kairuz *et al*.[11] It created the product with 495 base pairs (Fig. 2). By a DNA thermal cycler (Eppendorf, Hamburg, Germany), amplicons were amplified as follows: Initial denaturation for 5 min at 95.00 ˚C, pursued by 32 amplification cycles (95.00 ˚C for 30 sec, 55.50 ˚C for 30 sec, 72.00 ˚C for 1 min), and a final extension cycle (72.00 ˚C for 5 min). For amplification, a total volume of 25.00 µL of reaction mixture containing 1.00 µL of reverse primer (10.00 µM), 1.00 µL of forward primer (10.00 µM), 2.50 µL of template DNA, 8.00 µL of distilled water, and 12.50 µL of Taq DNA Polymerase 2X Master Mix (Amplicon, Odense, Denmark) with 0.20 mM of

deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), 1.50 mM magnesium chloride ($MgCl_2$), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP) was used. A PCR product of 8.00 µL in 1.00% agarose gel (w/v) with Tris-acetate-EDTA (TAE; 100 mM Tris HCl [pH 9.00], 40.00 mM EDTA) containing 3.00 µL of safe staining (Sinaclon, Tehran, Iran) was electrophoresed and by transillumination under ultraviolet (Uvidoc HD6; Uvitec, London, UK) was observed. The size of the amplified products was appraised through comparison with a DNA ladder of 100 bp (Sinaclon).

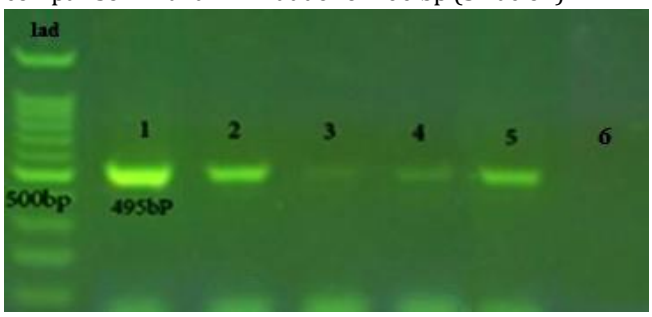

Fig. 2. The PCR products were electrophoresed in agarose gel by cytochrome P-450 primer which was set to detect *Candida spp.* 1-4: positive PCR product with a size of 495 bp of *Candida spp.*; 5: positive control; 6: water as negative control; Lad: 100-bp molecular marker.

Sequencing and phylogenetic analysis. The PCR products with the forward and the reverse primer were sent to the Bioneer sequence service (Bioneer, Daejeon, Korea) for sequencing. After converting the sequences to FASTA format, they were aligned and were recognized by searching databases using online system of local alignment tools (BLAST) on the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm. nih.gov). Clustal W method using MEGA Software (version 6.0; Biodesign Institute, Tempe, USA) and SnapGene (version 3.2.1; GSL Biotech LLC, Boston, USA) were used to compare our sequences with other related sequences in NCBI. With MEGA 6 software, by the neighbor-joining algorithm and the Jukes-Cantor distance model, the phylogenetic trees were created according to the nucleotide sequences of the cytochrome P-450 L1A1 gene (Fig. 3).

Results

Isolation and microscopic examination. In total, 19.00% of pigeons studied were positive by the culture (Fig. 1A) and microscopic test (Fig. 1B), and 22 *Candida* isolates were isolated from these pigeons. The status of pharyngeal or cloaca infection of these 19 pigeons is summarized in Table 1. Nine percent of pigeons, sampled through only cloacal swabs were positive in the culture method as well as the microscopic examination. The most prevalent clinical symptoms in these pigeons were diarrhea resistance to antibiotics. A 7.00% of pigeons, sampled through only pharyngeal swabs were positive by culture and direct examination. The most prevalent clinical symptoms in these pigeons were white mucous plaques in their mouth, crop stasis, regurgitation, and vomiting. A 3.00% of pigeons, sampled through both cloacal and pharyngeal swabs were positive. The most prevalent clinical symptoms in these pigeons were diarrhea which was resistant to antibiotics, crop stasis, white diphtheritic membranes in their mouths, regurgitation, and vomiting.

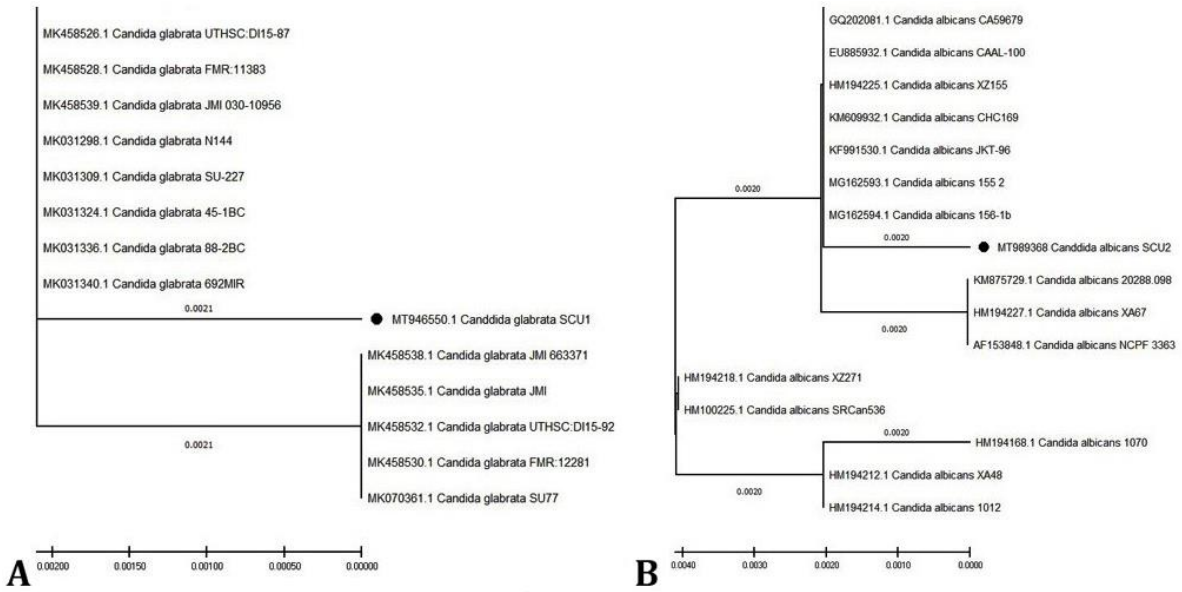

Fig. 3. A) The Neighbor-Joining algorithm MEGA Software was used to compare the phylogenetic position of the present *C. glabrata* based on cytochrome P-450 lanosterol-a-demethylase (L1A1) gene sequences (MT946550) with other related taxa available in the GenBank® database. **B)** The Neighbor-Joining algorithm MEGA Software was used to compare the phylogenetic position of the present *C. albicans* based on cytochrome P-450 L1A1 gene sequences (MT989368) with other related taxa available in the GenBank® database.

Table 1. Status of positive pigeons by the type of samples and the most prevalent clinical signs.

Samples	No. (%)	The most prevalent clinical signs
Cloacal swabs	9 (9.00)	Diarrhea resistant to antibiotic
Pharyngeal swabs	7 (7.00)	White mucous plaques in their mouth, crop stasis, regurgitation, vomiting
Pharyngeal and cloacal swabs	3 (3.00)	Diarrhea resistant to antibiotics, white diphtheritic membrane in mouth, regurgitation, vomiting
Total infected pigeons	19 (19.00)	-

PCR and molecular identification. All 22 isolates were chosen for molecular diagnosis of the organism, and DNA was extracted from these samples. All 22 suspected samples were positive by PCR (Fig. 2). Nine isolates from nine diseased pigeons, which were sampled through only cloaca swabs were positive by PCR. Seven isolates from seven diseased pigeons, which were sampled through only pharyngeal swabs were positive by PCR. Six isolates from three diseased pigeons (three cloacal swabs and three pharyngeal swabs), which were sampled through both cloacal and pharyngeal swabs were positive. Altogether 22 isolates were proved as *Candida* species.

Gene sequencing of *Candida spp*. Two PCR products were sequenced and according to the gene sequences, these sequences were *C. albicans* and *Candida glabrata.* All sequences obtained in the current study were sent to GenBank® under accession numbers MT946550 and MT989368. By the neighbor-joining method and the Jukes-Cantor distance model, phylogenetic trees were built (Fig. 3). Bootstrap support was evaluated with 1,000 duplicate analyses. The results revealed a 99.54% accordance when compared with other sequences of *C. glabrata* (MK031340.1, MK031336.1, MK031324.1, MK031309.1, MK031298.1, MK458528.1, and MK458539.1) which were formerly deposited in GenBank® from the Netherlands and Spain. The results revealed a 99.78% accordance when compared with other sequences of *C. albicans* (MG162594.1, MG162593.1, KF991530.1, KM609932.1, and MT081010.1) which were formerly deposited in GenBank® from Colombia, Indonesia, China, and Sudan.

Discussion

Many studies suggest that contamination of public places with pigeon feces can be a source of infections such as *E. coli* O157, Salmonella, and Campylobacter which they could affect public health. [5,12] Transmission of pathogenic yeasts through pigeon feces is important and has been studied worldwide. According to Wu *et al*., the presence of eight different yeast genera such as *Cryptococcus*, *Candida,* and *Rhodotorula* in pigeon feces was confirmed in Beijing, China.[13] Costa *et al*. in Brazil, also reported that *Cryptococcus*, *Candida*, and *Rhodotorula* species are often found in pigeon feces. *Candida* species isolated from pigeon feces are considered pathogenic to humans and animals, especially in immunodeficient individuals.[14] Other reports from the Canary Islands,[4] Egypt,[15] India,[16] and Sweden,[17] focused on the importance of pigeons' feces as a potential source of pathogenic yeast. Pigeons are one of the most important carriers of Candida species.[18,19] According to Costa *et al*., several species (*Candida guilliermondii, C. albicans, Candida kefyr*) were isolated from cloacal samples for the first time, but most of the isolated yeasts were *C. guilliermondii* and *C. albicans*.[14] In the present study, 9.00% of pigeons sampled through only cloacal swabs were positive in the culture as well as the wet mount test, and two species *C. albicans* and *C. glabrata* were identified by sequencing. The detection of *C. glabrata* can be important as this species has been isolated from immunocompromised patients. Although *C. albicans* is the most common agent responsible for infection in various forms of candidiasis, some other *Candidae*, including *C. glabrata, C. tropicalis, C. krusei, C. guilliermondii, C. parapsilosis* more or less isolated from patients. The importance of *non-albicans* species such as *C. tropicalis* and *C. glabrata* in recent years due to their resistance against antifungal drugs has increased. It increases the prevalence of these *non-albicans* species.[7,20,21] Long-term use of antibiotics can weaken the bird's bacterial flora and overgrow Candida. Other factors such as weakened immune system, environmental stress, various infectious diseases, malnutrition, and keeping a large number of birds in a limited space are involved in the formation of the disease.[8] In the present study, many of these predisposing factors were present in the history taken from the birds. In a study of Hasenclever and Kogan, *C. albicans* was reported in 44.00% of upper gastrointestinal specimens and 6.00% of lower gastrointestinal specimens in pigeons.[9]

Similarly, in this study, *Candida spp.* was proved in oro-pharyngeal swab samples (upper gastrointestinal tract) and cloacal swab samples (lower gastrointestinal tract). In a study by Jang *et al*., the prevalence of fungal diseases in pigeons' feces was confirmed at 41.20% of the samples.[22] In the above study, fresh pigeons' feces were collected from 21 locations in Seoul, and the diversity of fungal species was assessed, and *Candida spp.*, mostly *C. glabrata*, *C. famata*, and *C. albicans* were confirmed.[22] In accordance with the results of Jang *et al.*, the presence of *C. glabrata*, and *C. albicans* was also confirmed in the current study.

In the study by Soltani *et al.*, similar to the results of the present study, *C. albicans* was isolated from pigeon feces [23], but in the study by Soltani *et al.*, unlike the present study, only healthy pigeons were sampled.

In a study by Rosario Medina *et al.*, pigeons' feces were evaluated as a source of *Candida species*.[19] They collected 174 dropping samples, 331 cloacal samples, and 331 crop samples from healthy pigeons. The most common yeast isolated was *C. guillermondi*, which was detected in 30.46%

of crop samples, 24.36% of cloacal samples, and 49.37% of dropping samples. Also, *C. albicans* was often isolated from pigeons' feces.[19] The results of Rosario Medina *et al.* suggest that pigeons' feces can be vectors and repositories of *Candida spp*. It should be noted that in the current study, candidiasis was proved in 19.00% of sick pigeons by direct microscopic observation, culture, and PCR method.

The reason for the discrepancy between the results of the current study and the results of Rosario Medina *et al.* may be because of the difference in the sample size. In the current study, only sick pigeons were checked out, and the number of sick pigeons was low, but in the study by Rosario Medina *et al.*, specimens were collected from healthy pigeons, and their sample size was large.

The contradiction of the results of the current study with some of the mentioned studies may be due to the following factors: geographical distribution of *Candida spp.*, sample size, the general health status of birds, herd management practices, and methods used to identify *Candida spp*. In all of the mentioned surveys, research has been performed on healthy birds and opportunistic *Candida spp.*, while the current study attempted to examine only specimens of sick pigeons suspected to candidiasis and to investigate only pathogenic *Candida spp.*, so the number of samples in the current study was less than in other studies. In the present study, by culture method, the total number of positives (19 samples out of 100 samples), was low. This low percentage of positive cases may be because candidiasis was not the cause of clinical manifestations, and other diseases caused such clinical signs in pigeons.

For example, diseases like hypovitaminosis A, wet pox, and trichomoniasis may lead to the formation of white diphtheritic membranes in the mouth, and Entero-bacteriaceae infections like colibacillosis or salmonellosis may cause diarrhea and the appearance of these clinical signs has nothing to do with candidiasis. In the current study, based on the visual assessment by researchers on the growth density, samples comprising pure candida colonies which were grown in zones 3 and 4 of the plate were evaluated severely infected and purified for subsequent evaluation. Samples that grew just in the first and second zones of the plate were evaluated as yeasts that were purely microbial flora (opportunistic *Candida spp.*) and were not subsequently evaluated because this survey aimed to evaluate only patient specimens suspected of candidiasis and to examine pathogenic *Candida spp.* Therefore, with this method, the positive cases by culture method were low. In the survey by Abulreesh *et al*., amazingly, *Candida* spp. was approximately absent, and only one isolate (2.10%) was detected as *C. glabrata* in pigeons' feces.[24] Similar to that study, in the current study *C. glabrata*, was proved in pigeons' feces, but inconsistent with the study of Abulreesh *et al*., in this study *C. albicans* also was proved.

In the present study, 19.00% of pigeons, with oral lesions or gastrointestinal problems suspected of having fungal diseases, introduced to the Department of Avian Medicine, Ahvaz, Iran were positive by culture and direct examination (wet mount), and 22 *Candida* isolates were isolated. All isolates were chosen for molecular diagnosis of the organism, and DNA was extracted from these samples. All 22 suspected isolates were positive by PCR. So, in the present study, *Candida spp.* was detected in 100% of the isolates by PCR. The present study showed that the most prevalent clinical symptoms in positive pigeons included diarrhea resistant to antibiotics, crop stasis, white diphtheritic membranes in their mouths, regurgitation, and vomiting. Thus, in birds with these symptoms, candidiasis should be considered as one of the possible causes, and taking the necessary and timely preventive and therapeutic measures is vital.

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Conflict of interest

The authors declare that there is no conflict of interest.

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