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Migratory Birds as a Potential Reservoirs of Cryptococcus Neoformans

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ABSTRACT: Migratory birds can become long-distance vectors for a wide range of microorganisms. The objective of the present study was to investigate the presence of Cryptococcus neoformansin the cloacae, crop and nasal cavity of migratory birds in order to assess their role as potential reservoirs and/or mechanical vectors of human and animal cryptococcosis. A total of 700 samples (cloacae, crops and nasal secretions) of 300 wild migratory birds (with the permission of the local Department of Environment) were collected and inoculated on Niger seed agar (NSA), incubated for two weeks at 30 ºC and daily observed for the presence of brown yeast colonies, which is presumptive for C. neoformans. The species identification was confirmed using conventional and molecular methods. Out of 700 samples, 4 samples (0.6%) from cloacae of 2 Anascrecca (2 cases), Anasplatyrhychos (1 case) and Fulicaatra (1 case) were positive for C. neoformans. To the best of our knowledge, due to low isolation rate of C. neoformansfrom cloacae, crops and nasal secretions, transmission from these samples could be as a minimal risk factor for human and animal cryptococcosis, unlike the dry excreta of wild pigeons.

Key words: Migratory birds, Cryptococcus neoformans, Wild birds, Cloaca, Crop, Nasal secretion

INTRODUCTION

Over the past decades, a growth in the prevalence of cryptococcosis has been documented among the immunocompromised individuals (Cafarchia et al., 2006a).

Cryptococcus neoformansand C.gattii are the known pathogenic agents involved in the incidence of cryptococcosis in humans and warm-blooded animals around the world (Blaschke-Hellmessen 2000). Following an analysis of the C. gattii/C. neoformans species complex, Hagen et al. concluded that C. neoformans var. grubiiand C. neoformans var. neoformans need to be regarded as separate species. They also found five other species within the mentioned complex (Hagen et al., 2015). According to Meyer et al., among the various Cryptococcus,C. neoformanshas the highest global occurrence (63%) (Meyer et al., 2011). It has been commonly isolated from the droppings of different bird species, including pigeons, doves, ducks, blue peafowls, parrots (Filiu et al., 2002.), passerines, raptors, and birds in a commercial aviary (Cafarchia et al., 2006a). C. deneoformans (formerly C.neoformansvarneoformans) also accounts for 5% of the collected isolates and is widely seen in Europe. Although less common than C. neoformans, C. deneoformans have been isolated from soil samples, birds and their excretions, cats, dogs, and trees (Mitchell et al., 2011).

While C.gattii complex is believed to occur only in tropical and subtropical areas, the species has been recently detected in Southern Italy (Montagna et al., 2002). C. gattii is generally isolated from Eucalyptus trees (Pfeiffer and Ellis 1992) and their decaying wood (Granados and Castaneda 2005). However, reports of its isolation from bird droppings have also been published (Abegg et al., 2006). This species is capable of infecting even immunocompetent individuals (Chen et al., 2000). On the other hand, C. neoformansvar. neoformans and C. neoformansvar. grubiihave been

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widely collected from the soils and bird excretions throughout the world (Ruiz et al., 1981).

While cryptococcosis is not typically transmissible from one animal to other animals or humans, in one case, an immunocompromised individual was reported to acquire the disease from the feces of a healthy-looking pet bird (Jacob and Cantor2011). Contact with bird excretions, particularly those from domestic pigeons (Granados and Castaneda 2005, Kobayashi et al.,2005), Psittacine and Passerine birds (Filiuet al., 2002., Abegg et al., 2006, Lugariniet al., 2008a) is generally considered as the main cause of the disease in such cases (Kobayashi et al., 2005). Nevertheless, the inability of Cryptococcus species to grow at birds' body temperature and to survive within the birds' intestinal tract, their presence in bird habitats is rarely associated with infections and the incidence of the clinical disease in birds (Mitchell and Perfect 1995, Casadevall and Perfect 1998, Filiuet al., 2002.).

Yeasts and yeast-like fungi, e.g. Cryptococci, isolates have been collected from migratory birds (Malik et al., 2003).Since migratory birds travel long distances and colonize in various urban and suburban areas (Rosario et al., 2005, Rosario et al., 2010, Foti et al., 2011), they can play a role in disease transmission and increase the zoonotic potential of infections. Wild bird-borne infections can either be localized or involve humans and animals residing in short and long distances. Meanwhile, the transmission of infectious agents from wild birds to humans over a long distance has the greatest epidemiological significance (Tsiodraset al.,2008). Although such cases of transmission are theoretically possible, they are not well confirmed by scientific evidence. However, Cryptococci are ubiquitous pathogens which can be indirectly transmitted to humans from wild pigeons (Columbidae). These pathogens may even cause clinical infections, particularly in patients with immunodeficiency (Fessel 1993).

SinceCryptococcus is commonly present in wild pigeons(Columbidae) (Fessel 1993, Decostere et al., 2003, Malik et al., 2003, Raso et al., 2004, Tintelnot and Losert 2005), Psittacine birds (Psittaciformes), starling (Sturnidae),in Europe, South America, Asia (Tsiodras et al., 2008), the present study was the first to assess the role of migratory birds as potential reservoirs and/or mechanical vectors of C. neoformansand C. gattiiand their significance in the transmission of infections to humans and animals in Mazandaran province, Northern Iran. We hypothesize that wild birds' crop, cloacae, and nasal cavity may act as a potential reservoirs for the mentioned pathogens.

MATERIALS & METHODS

Sampling procedures:A total 300 Carcasses of wild migratory birdswere legally captured with nets trap in January 2012 and January 2013 at the Mazandaran Province Northern Iran, based on normal licensing procedure(http://www.iew.ir/1394/08/12/42055) for hunting wild birds by Iranian Environmental Protection Agency (EPA). The cloaca, crop and head of the hunted wild bird from 8 different bird species including Anasclypeata, Anasstrepera, Anasacuta, Anasplatyrhychos, Anascrecca, AnasPenelopeAythyafuligula and Fulicaartaweredissected .A total of 700 samples (300 cloacae, 300 crop and 100 head transported in cold boxand processed within 2 hours.

Mycological culture and identification procedures:Freshcloacacontent (0.5 g) were suspended in 4.5 ml sterile physiological saline (0.9%) with chloramphenicol (100 mg/l), and the mixture was shaken for 30 min at 100 rpm. and allowed to stand for 15min (Foti et al., 2011).

One hundred microliters of each cloaca sample was streaked on Niger seed agar (NSA; Guizotiaabyssinica, 50g; dextrose, 1g; KH2PO4,1g; creatinine, 1g; agar 15g, chloramphenicol, 1g; and 1000 ml dH2O), for the selective isolation of C. neoformans(Lugarini et al., 2008a). After removing the crops contents, using cottonsterileswabs the materials from surface were collected. The birds' heads were opened by a scalpel and the nasal secretion were taken using swab. Eachswab samples were inoculated on NSA, separately.

The NSA medium were incubated at 30 ^{o}C for 15 days and daily observed for the presence of brown yeast colonies. Presumptive colonies of C. neoformans were selected and sub cultured on Sabouraud dextrose agar(Difco, Milano, Italy) with chloramphenicol for single colonies. The isolates were identified to the species level based on their morphological and biochemical characteristics (Franzot et al., 1997, Takahara et al., 2013). Briefly,each positive colonies were examined microscopically using nigrosinstaining for detecting capsules. Identification was based on microscopic features, cycloheximide sensitivity (0.1%), growth at 37 ^{o}C and urease production (Takahara et al., 2013).

C. neoformanswas detected if the L- canavanine glycine-bromothymol blue agar medium (CGB) remained yellow-green in color after 48 h at 27 ^{o}C (Klein et al., 2009).

DNA isolation, amplification and sequencing ITS regions:Genomic DNA was extracted by using a (glass bead - phenol chloroform) method as previously described (Yamada et al., 2002). For accurate identification of the C. neoformans isolates, the polymerase chain reaction (PCR) was developed to amplify the internal

transcribed spacer regions (ITS1 and ITS2) rRNA using a primer pairs, ITS1 (5´TCC GTA GGT GAA CCT TGC GG 3´) and ITS4 (5´TCC TCC GCT TAT TGA TAT GC 3´), (Katsu et al., 2004, Korabecna 2007). PCR products were sequenced using BigDye Terminator V3.1 on the ABI 3730XL DNA Analyzer using Bioneer sequence service, (Bioneer, Daejeon, Korea).

The PCR products were sequenced with forward (ITS1) and reverse primer (ITS4). After conversion the sequences to a FASTA format, they aligned and species were identified by searching databases using the online basic local alignment search tool (BLAST) system at the website of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

RESULTS& DISCUSSION

Out of 700 samples, 4 samples (0.6%) from cloacae of Anascrecca (2 cases),Anasplatyrhychos (1 case) andFulicaatra(1 case)were positive for C. neoformans. The fungal ITS region of rRNA gene in these samples were amplified and yielded a 560 bp fragment. (Fig. 1). Sequence analysis of PCR amplicons analysis using BLAST revealed that all of the amplified sequences had 99% identity with C. neoformans (formerly C. neoformans var. grubii)reference sequences.The sequences were submitted to the NCBI GenBank and assigned with accession numbers KT585710 and KT696599-601.

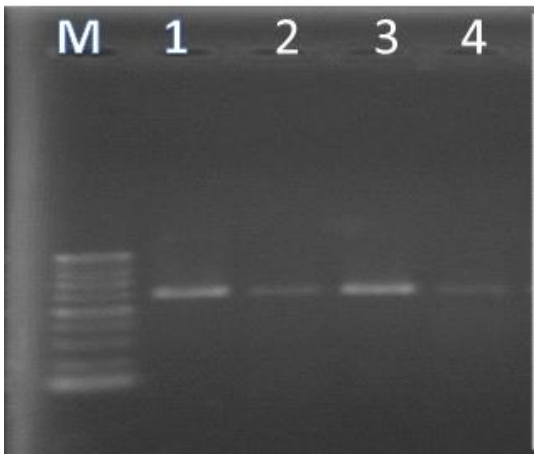

Fig.1. PCR products of C. neoformans with ITS1 - ITS4 primers on 1.5% agarose gel. Line 1;100 bp molecular marker, lines 1 to 4; isolates of C. neoformans

To the best of our knowledge, this is the first report representing the occurrence and distribution of C. neoformans strain in cloaca, crop and nasal secretion of migratory birds using conventional and molecular methods, in Iran.

As a pathogen for humans and animals, C. neoformanshas unique epidemiological and environmental features.Over the past decade, some studies have noted the role of pigeons in increasing the importance of C. neoformansin creating cryptococcosis(Maliket al., 2003, Granados and Castaneda 2005, Kobayashi et al., 2005, Lugarini et al., 2008a).Using molecular techniques, many studies have shown that pigeons are not the only effective species spreading C. neoformansin cities, but also many other wild (Ramirez et al., 1976, Mattsson et al.,1999) and migratory (Foti et al., 2011) birds have been identified as sources for the yeast, contributing to distribution of these pathogens.

In this regard, numerous investigations have been conducted on bird feces both in Iran (Khosravi 1997, Mikailie 2001, Nasr Isfahani et al., 2001, Zarrin et al., 2010, Hedayati et al., 2011, Agha Kuchak Afshari et al., 2012, Soltani et al., 2013) and abroad (Caicedo et al., 1999, Nosanchuk et al., 2000, Mancianti et al., 2002, Nishikawa et al., 2003, Raso et al., 2004, Rosario et al., 2008); however, there is a dearth of research on the digestive tract of these birds with a limited focus on feral pigeons (Ramirez et al., 1976, Mattsson et al., 1999).

The present study was a pioneer research project in Iran aiming at molecular determination and identification of C. neoformansand Cryptococcus species isolated from the cloacae and crop of migratory birds. The current study revealed the low occurrence (0.6%) of C. neoformans in the cloacae sample of birds, which is in line with many other studies noting the hardship of isolating C. neoformans from the digestive tract of birds (Mattsson et al., 1999, Rosario et al., 2005, Cafarchia et al., 2006a, Cafarchia et al., 2006b, Lugarini et al., 2008a, Lugarini et al., 2008b, Rosario, et al., 2010, Foti et al., 2011).Rosario et al. (2005) isolated only 1.81 % C.neoformans var.neoformans from cloacal swabs of pigeons(Rosario et al., 2005).

Among several studies conducted on the crop of migratory birds (Cafarchia et al., 2006b) and pigeons (Lugarini et al., 2008b), no positive cases of C. neoformans and C. gattii were isolated from the digestive tract. However, C. neoformansvar. grubii was isolated from cloacal swabs of 4.8% of birds of prey (Cafarchia et al., 2006a). Mattson et al., found no cases of C. neoformans in pigeon cloacal swabs; however, they observed other medically important yeasts, such asDebarymyceshanssenii,C. laurentiiandC. uniguttulatus(Mattssonet al., 1999).

In a research, 19 fresh fecal samples of migratory birds were examined, among which C. neoformans was just isolated from the culture of one pooled fecal sample (Foti et al., 2011).It seems that one of the reasons for the low isolation of C. neoformans from the cloacae

and crop is the weak growth of the yeast in the body of birds due to high body temperature and probably would be a result of high concentration of ammonia in fresh feces in cloacae (Cafarchia et al., 2006b). In addition, various factors, such as Zygomycota overgrowth, may affect the isolation of the yeast leading to false negative results in the medium (Granados and Castaneda 2005, Kobayashet al., 2005). Some researchers stressed the need to conduct studies to determine the relationship between virulence and pathogenicity of the yeast and the colonization thereof in the cloacae and crop of birds (Krockenberger et al., 2002) to further clarify how C. neoformans lives in the body of different species of birds (Lugarinet al., 2008b).

C. neoformans can be presented in very small size outside of a bird's body and easily moved to different areas, such as humans' place of residence, by the wind (Chee and Lee 2005). Moreover, birds carrying C. neoformans can spread these pathogenic fungi in urban rest areas during the flight.This is particularly important in terms of the exposure of children, the elderly as well as the immunocompromised patients who are more prone to develop this opportunistic infection (Cafarchia et al., 2006a).

Finally, the results of this study showed that the occurrence of C. neoformans isolated from the digestive tract of wild birds was extremely low and no cases were found in the upper respiratory tract of migratory birds.C. neoformans, as an endo-saprophytic, does not live in the digestive tract of wild birds and these birds may only contribute to transmit the yeast in the environment, and also that they find a favorable condition for their development in birds' excreta (Lugarini et al., 2008b). Additionally, the yeast had few little aggressive capacity in bird species examined in this study.

The occurrence of C. neoformans var. grubii (serotype A) in four samples was not surprising, because these are the most common species in the world that can be observed in birds, especially pigeons, and their habitats and waste, contaminated soil and decaying wood (Gugnani et al., 2005, Litvintseva et al., 2006, Viviani et al., 2006, Hiremath et al., 2008, Randhawa et al., 2011).Moreover, in many human and veterinary clinical cases worldwide, the most common type of C. neoformans is grubii(Casadevall and Perfect1998, Viviani et al., 2006).The result of the present study is consistent with the results of many other studies stating that it is difficult to isolate C. neoformans from the digestive system of birds(Mattsson et al., 1999, Cafarchia et al., 2006b, Lugarini et al., 2008b).

CONCLUSIONS

Although the description of the direct or indirect role of wild birds in transmission of infection to humans was not fully possible, this study yielded some remarkable results. First, due to low isolation rate of C. neoformans from cloaca, crop and nasal secretion there is no conclusive evidence indicating the special role of wild birds in the direct transmission of infection to humans. In theory, cryptococcosis can be transmitted from wild and migratory birds, though the scientific basis for this issue is rather skeptical. Appropriate understanding of bird migration routes and also conducting further research using advanced molecular techniques can be effective in determining the prevalence, and transmission of this pathogen by different species of migratory birds. To the best of our knowledge, due to low isolation rate of C. neoformans from cloacae, crop and nasal secretion, transmission from these samples could be as a minimal risk factor for human and animal cryptococcosis, unlike the dry excreta of wild pigeons.

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