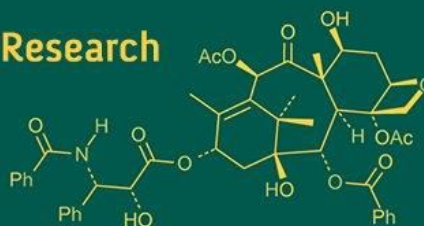


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Navdeep Kaur
Department of Zoology,
Punjab Agricultural
University, Ludhiana, Punjab,
India

Tejdeep Kaur Kler
Department of Zoology,
Punjab Agricultural
University, Ludhiana, Punjab,
India

Charanjit Kaur Dhanju
Department of Zoology,
Punjab Agricultural
University, Ludhiana, Punjab,
India

Pathogenic fungal diversity in excreta of free living wild birds in agro ecosystems of Ludhiana

Navdeep Kaur, Tejdeep Kaur Kler and Charanjit Kaur Dhanju

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Abstract

The excreta samples of six free living bird species (blue rock pigeon, rose ringed parakeet, common myna, house crow, cattle egret and red wattled lapwing) in the agroecosystems was screened for the presence of pathogenic fungi (moulds and yeast). The isolated moulds were identified to be *Aspergillus fumigatus*, *A. niger*, *A. flavus*, *Alternaria* sp. and *Geotrichum* sp. The prevalence and population density of these moulds varied across the bird species. *A. fumigatus*, *A. flavus* and *Alternaria* sp. were found to be commonest species occurring in 60% of the samples followed by *A. niger* (40%) and *Geotrichum* sp. (20%) in excreta of blue rock pigeon with highest population density of *Alternaria* sp. (followed by *A. fumigatus*, *Geotrichum* sp., *A. niger* and *A. flavus*). The house crow excreta showed 100% prevalence of *A. fumigatus* followed by *A. niger* (60%) and *A. flavus* and *Alternaria* sp. (20% each) and carried the highest population density of *A. fumigatus* and *Alternaria* sp. followed by *A. niger* and *A. flavus*. In cattle egret excreta, *A. fumigatus* showed 100% prevalence, but other moulds were isolated from only one sample with the highest population density of *Geotrichum* sp. followed by *A. fumigatus*, *A. flavus*, *Alternaria* sp. and *A. niger*. The single excreta sample revealed maximum population densities of only *A. fumigatus* in rose ringed parakeet; *A. fumigatus* followed by *A. niger* and *A. flavus* in red wattled lapwing; *A. flavus* as well as *Geotrichum* followed by *A. fumigatus* in common myna. The prevalence of *A. fumigatus* was highest (86.7%) followed by *A. niger*, *A. flavus* (46.7%), *Alternaria* sp. (33.3%) and *Geotrichum* sp. (20%) in all the analyzed excreta samples. The yeast *Cryptococcus neoformans* was isolated from fresh and dry excreta of blue rock pigeon and fresh excreta of house crow only. The prevalence of pathogenic fungi in the excreta of these bird species indicates that bird excreta may be a source of infection to human beings as well as to birds themselves.

Keywords: Free living birds, excreta, moulds, *Cryptococcus*, pathogens

Introduction

Birds play an essential role in agroecosystems, by providing a variety of functions, including predators, pollinators, scavengers, seed dispersers, pests controller, nutrient cycling, ecosystem engineers, and many others thus benefiting both the environment and agricultural productivity. The complex relationships between birds and microorganisms are increasingly becoming the subject of ecological research (Maul *et al* 2005) [1]. The pathogenic and allergenic microbial species can act to reduce fitness, making individuals more susceptible to competition and predation, while severe infections/reactions are significant causes of mortality (Nuttall 1997) [2]. Fungi are important causes of diseases in wild birds and other species. The common birds such as pigeons harbor a number of fungal species which are of potential health hazard and poses a risk to humans (Joshi *et al* 2015) [3]. The various filamentous fungi such as *Aspergillus* sp. (31.1%), *Mucor* sp. (20.2%), *Penicillium* sp. (9.5%) and *Fusarium* sp. (6.7%) was isolated from pigeon excreta samples (Cermeno *et al* 2006) [4]. Similarly, *Aspergillus*, *Rhizopus*, *Penicillium*, *Mucor* and *Fusarium* species was isolated from *Leptoptilos crumeniferus* (marabou stork) droppings in Kenya (Nyakundi and Mwangi 2011) [5]. Aspergillosis is a complex of diseases which is caused by fungal pathogens such as *Aspergillus fumigatus*, *A. flavus*, *A. terreus*, and *A. niger* and most of the infections was caused by *A. fumigatus* in humans and avian species (Melo *et al* 2020, Kanauji *et al* 2023) [6, 7]. Recently, *Aspergillus fumigatus* has been listed as the third most critical pathogen by the World Health Organization (WHO) which plays a significant role in the prevalence of aspergillosis (Khan *et al* 2024) [8].

Corresponding Author:
Navdeep Kaur
Department of Zoology,
Punjab Agricultural
University, Ludhiana, Punjab,
India

The spores of *Aspergillus* when inhaled can cause serious and often fatal infections in a wide variety of captive and free-roaming wild birds (Arné *et al* 2021) [9]. Aspergillosis is a major cause of morbidity and mortality in birds (Tell 2005) [10]. A unique feature of avian aspergillosis is the presence of reproductive phases of *Aspergillus* sp. in tissues which is due to presence of cavernous air sacs, the warm core body temperature of birds (range= 38-45 °C) that provide a nutrient rich environment. The number of colonies for establishing a definitive diagnosis is usually between one and four; the recovery of large numbers carries a severe prognosis.

Cryptococcus neoformans is opportunistic basidiomycete yeast that causes cryptococcosis, a life threatening infection. This yeast has 3 varieties known as *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D), and *C. neoformans* var. *gattii* (serotype B and C). Cryptococcosis, caused by *C. neoformans* primarily affects the nervous system, leading to meningoencephalitis in humans and this infection can be fatal without proper treatment in some of the cases. An Inhalation or direct contact with spores of *C. neoformans* from bird droppings poses a major health risk to humans (Al-Temimay and Hasan 2016) [11]. The most important source of *C. neoformans* var. *neoformans* throughout the world is pigeon excreta and other avian droppings (Zarrin *et al* 2010 and Nawange *et al* 2011) [12, 13]. Many bird species play an important factor in fecal contamination of drinking water sources and agricultural crops. Keeping this in view, it was planned to isolate and identify the pathogenic fungi (moulds and yeast) from the excreta of free living wild birds commonly found in agro ecosystems.

Materials and methods

Study area

Fresh excreta of different bird species (blue rock pigeon, *Columba livia*; rose ringed parakeet, *Psittacula krameri*; common myna, *Acridotheres tristis*; house crow, *Corvus splendens*; cattle egret, *Bubulcus ibis* and red wattled lapwing, *Vanellus indicus*) was collected from their roosting, foraging and nesting sites in agricultural crop fields and residential areas of Punjab Agricultural University (PAU) located in the main campus of PAU (30° 54' N latitude and 75° 48' E longitude) and dairy farm of Guru Angad Dev Veterinary and Animal Sciences University (GADVASU) (30° 90' N latitude and 75° 80' E longitude).

Collection of excreta of birds

A total of 15 fresh excreta samples consisting of pooled pellets of six different bird species were collected. The five excreta samples were collected from commensal bird species such as blue rock pigeon and house crow (n=5). The two excreta samples of cattle egret (n=2) and one excreta sample each of rose ringed parakeet, common myna and red wattled lapwing (n=1) were collected. The two dry excreta samples of blue rock pigeon (n=2) was also collected from the above mentioned sites of collection. After collecting the excreta samples in sterile plastic bags with the help of spatula these were labeled appropriately with the source, time and date of collection and then stored at 4 °C till further processing.

Isolation and characterization of fungi

Ten gram of each sample was thoroughly mixed in 90 ml of normal saline. Aliquots (1ml) from serial logarithmic dilutions of each suspension were pipetted onto the surface of duplicate Petriplates containing sabouraud dextrose agar (SDA) supplemented with chloramphenicol (50 µg/ ml) to suppress bacterial growth and Staib's media. The inoculum was spread evenly with a sterile T-shaped glass applicator on petriplates. The petriplates were incubated at 28 °C for 5 days to 3 weeks. The mean colony count of moulds was determined and represented as colony forming units (cfu/ g) of each sample.

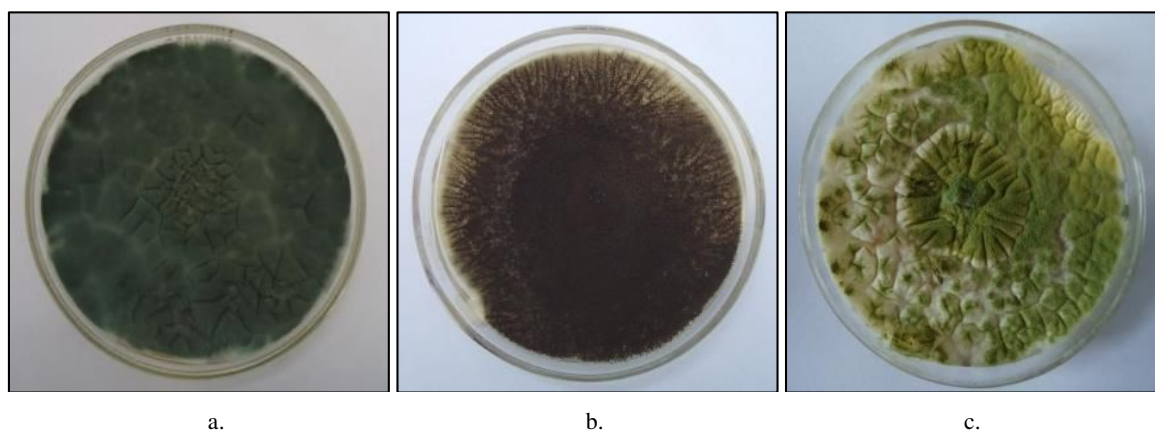
Identification criteria of fungi

The moulds isolated were identified by studying their colony and microscopic morphology as described by earlier published description (Cermeno *et al* 2006, Mishra 2010) [4, 14]. For the preparation of slide cultures, the technique of McGinnis (1974) [15] was used. The presence of brown-pink coloured colonies (*Cryptococcus neoformans*) appearing on Staib's medium were noticed and the isolated colonies were further tested by negative staining with Indian ink to study the presence of capsule.

Results and Discussion

Moulds

The isolated moulds in excreta samples of different bird species were identified to be *Aspergillus fumigatus*, *A.niger*, *A. flavus*, *Alternaria* sp. and *Geotrichum* sp. (Figure 1 and Figure 2). However, the excreta of different bird species varied in percent prevalence and population densities/ cfu/ g of these different moulds.



a.

b.

c.

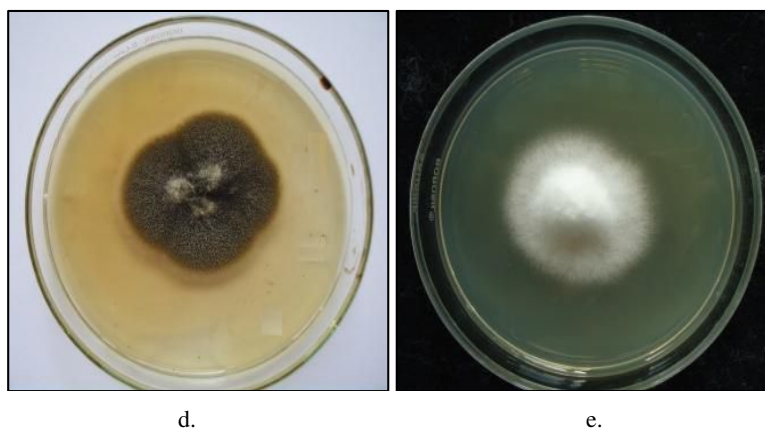


Fig 1: Morphology of mould colonies isolated from excreta of different bird species
a. *Aspergillus fumigatus* b. *Aspergillus niger* c. *Aspergillus flavus* d. *Alternaria* sp. e. *Geotrichum* sp.

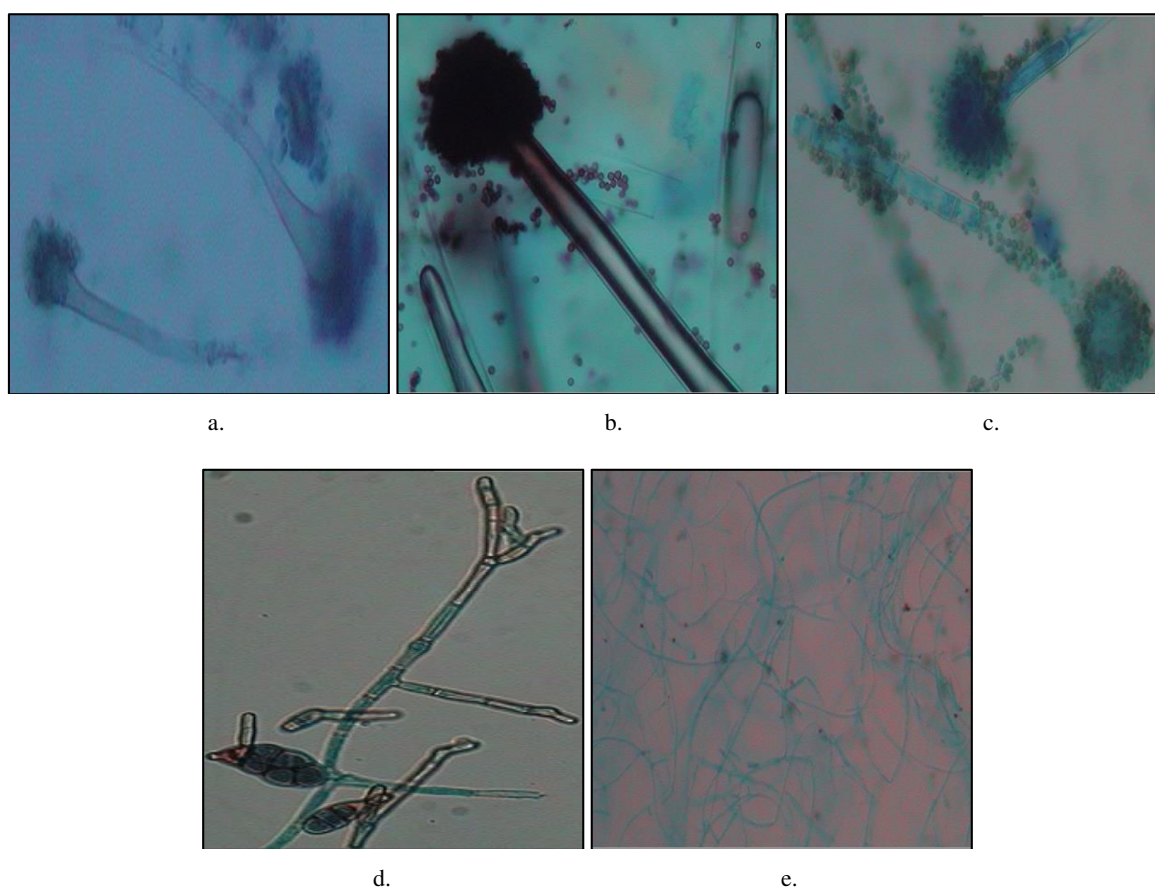


Fig 2: Slide culture preparation of mould colonies isolated from excreta of different bird species (Magnification 400 X) a. *Aspergillus fumigatus* b. *Aspergillus niger* c. *Aspergillus flavus* d. *Alternaria* sp. e. *Geotrichum* sp.

Three samples out of 5 samples of blue rock pigeon yielded one or more moulds (Table 1). *A. fumigatus*, *A. flavus* and *Alternaria* sp. were found to be commonest species occurring in 60% of the samples; followed by *A. niger* (40%) and *Geotrichum* sp. (20%) (Figure 3). Excreta of blue rock pigeon carried the highest population density of *Alternaria* sp. (11.7×10^5 cfu/ g) followed by *A. fumigatus* (6.7×10^5 cfu/ g), *Geotrichum* sp. (5×10^5 cfu/ g), *A. niger* (2.5×10^5 cfu/ g) and *A. flavus* (2×10^5 cfu/ g) (Table 2). *A. fumigatus* with the population density of 5×10^5 cfu/ g was present in one excreta sample of rose ringed parakeet. The population density of *A. flavus* and *Geotrichum* sp. was 5×10^5 cfu/ g and *A. fumigatus* was 2×10^5 cfu/ g in one excreta samples of common myna tested. A total no. of 5 samples of excreta of house crow were investigated for presence of

moulds. *A. fumigatus* showed 100% recovery from excreta of house crow, followed by *A. niger* (60%) and *A. flavus* and *Alternaria* sp. (20% each). The excreta of house crow carried the highest population density of *A. fumigatus* and *Alternaria* sp. (5.2×10^5 cfu/ g followed by *A. niger* (3×10^5 cfu/ g) and *A. flavus* (2.5×10^5 cfu/ g). A total no. of 2 samples of excreta of cattle egret was examined for presence of moulds and *A. fumigatus* was present in both the samples indicating 100% recovery whereas *A. niger*, *A. flavus*, *Alternaria* sp. and *Geotrichum* sp. could be isolated from only one sample indicating prevalence in 50% of the samples. The population density of *Geotrichum* sp. was higher (2×10^4 cfu/ g), followed by *A. fumigatus* (1×10^4 cfu/ g), *A. flavus* (2×10^3 cfu/ g), *Alternaria* sp. (5×10^2 cfu/ g) and *A. niger* (1×10^2 cfu/ g). The population density of *A.*

fumigatus was 8×10^6 cfu/ g followed by *A. flavus* (5×10^5 cfu/ g) and *A. niger* (6.5×10^4 cfu/ g) in one excreta sample of red wattled lapwing whereas *Alternaria* sp. and *Geotrichum* sp. could not be detected indicating that the excreta of red wattled lapwing carries only *Aspergillus* species and the population density of *A. fumigatus* was

highest in excreta of this bird species. The prevalence of *A. fumigatus* was highest (86.7%) followed by *A. niger* as well as *A. flavus* (46.7%), *Alternaria* sp. (33.3%) and *Geotrichum* sp. (20%) in the total excreta samples studied (Figure 4).

Table 1: Distribution of moulds in excreta of different bird species

Bird species	Total no. of samples examined	Number of positive excreta samples				
		<i>Aspergillus fumigatus</i>	<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Alternaria</i> sp.	<i>Geotrichum</i> sp.
Blue rock pigeon	5	3	2	3	3	1
Rose ringed parakeet	1	*	*	*	*	*
Common myna	1	*	*	*	*	*
House crow	5	5	3	1	1	ND
Cattle egret	2	2	1	1	1	1
Red wattled lapwing	1	*	*	*	*	*

ND refers to not detected

*Only one sample tested, thus no intraspecies comparison made

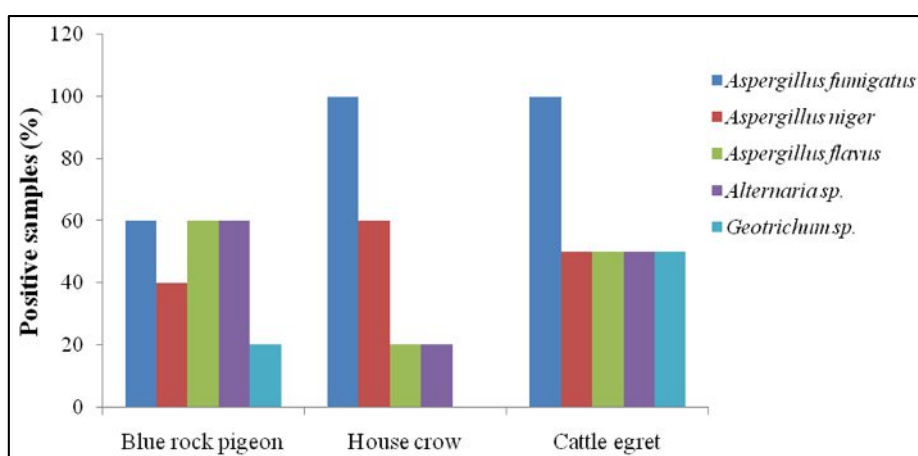


Fig 3: Different bird species excreta samples positive (%) for presence of moulds

Table 2: Population density (mean cfu / g of excreta) of moulds in different bird species

Bird species	<i>Aspergillus fumigatus</i>	<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Alternaria</i> sp.	<i>Geotrichum</i> sp.
Blue rock pigeon (n=5)	6.7×10^5	2.5×10^5	2×10^5	11.7×10^5	5×10^5
Rose ringed parakeet (n=1)	5×10^5	ND	ND	ND	ND
Common myna (n=1)	2×10^5	ND	5×10^5	ND	5×10^5
House crow (n=5)	5.2×10^5	3×10^5	2.5×10^5	5.2×10^5	ND
Cattle egret (n=2)	1×10^4	1×10^2	2×10^3	5×10^2	2×10^4
Red wattled lapwing (n=1)	8×10^6	6.5×10^4	5×10^5	ND	ND

ND refers to not detected

cfu refers to colony forming units

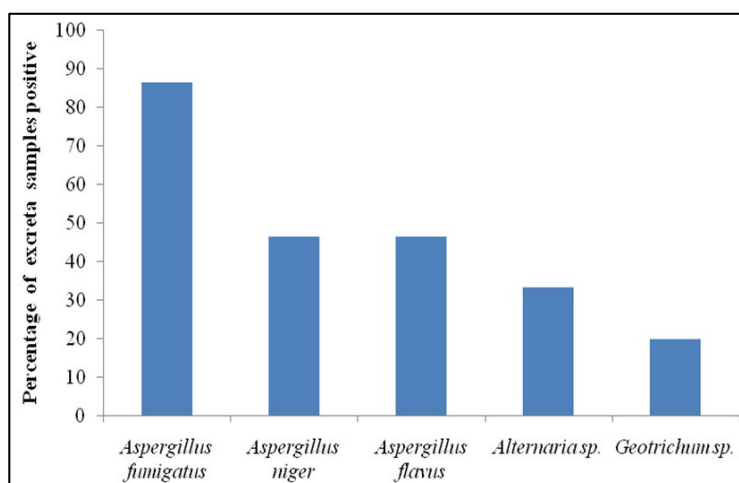


Fig 4: Percentage prevalence of moulds in excreta of all bird species

In the present studies, the excreta samples of all 6 species of birds contained pathogenic moulds. *Aspergillus* (especially *A. fumigatus*) was detected in all 6 species studied. House crow showed highest prevalence of *A. fumigatus* (100%) while the red wattled lapwing showed highest population density (8×10^6 cfu/ g) of *A. fumigatus* thus depicting the harmful exposure of their spores in environment which may affect the other birds as well as including humans. *A. fumigatus* has been determined as the most pathogenic among the causative agents of aspergillosis with widespread resistant spores present in the natural environment (Akan *et al* 2002) ^[16]. Aspergillosis is a respiratory tract infection caused by the genus *Aspergillus* of which *A. fumigatus* is the causative agent of infections in wild birds. Free-living wild birds become infected with *A. fumigatus* and other *Aspergillus* sp. causing aspergillosis which is a major cause of morbidity and mortality in birds. A wide variety of birds (waterfowl, gulls, crows, raven, raptors, songbirds, upland gamebirds, blackbirds, cowbirds, grackles, herons and shorebirds) have died of aspergillosis and probably all birds are susceptible to it. Young birds appear to be much more susceptible than adults (Friend 2001) ^[17]. Soltani *et al* (2013) ^[18] reported the occurrence of pathogenic fungi species such as *Penicillium* spp., *Aspergillus* spp., *Mucor* spp., *Rhizopus* spp., *Alternaria* spp., *Fusarium* spp., *Cladosporium* spp., and *Paecilomyces* spp. in the pigeon droppings collected from urban areas of Isfahan. Gómez-De-Anda *et al* (2023) ^[19] reported the occurrence of filamentous fungi genera such as *Mucor* spp., *Rhizopus* spp., *Penicillium* spp., *Paecilomyces* spp. and *Scedosporium* spp. with zoonotic potential in droppings of cattle egret collected from Mexico. The bird droppings have high concentrations of nitrogen compounds and nutrients due to which it provide fertile media for the growth and spread of various disease-causing microorganisms including pathogenic fungi (Mirhosseini and Khosrav 2023) ^[20]. Moreover, the organic debris accumulated in bird nests creates a unique

environment for development of various fungal groups (Błońska *et al* 2024) ^[21].

Yeasts

The pink colonies of *Cryptococcus neoformans* showing presence of capsules with Indian ink staining were isolated in both wet and dry excreta of blue rock pigeon and in wet excreta of house crow but was not detected in wet excreta of rose ringed parakeet, common myna, cattle egret and red wattled lapwing (Table 3, Figure 5). Earlier studies showed isolation of *C. neoformans* in old and dry excreta especially that of pigeon (Zarrin *et al* 2010, Nawange *et al* 2011) ^[12, 13]. Recently, it has been reported that *C. neoformans* showed a strong association with desiccated avian excreta (Chowdary *et al* 2012) ^[22]. The reason for the high occurrence of *C. neoformans* in avian excreta is believed to be due to a utilization of nitrogenous compounds such as uric acid and creatinine from bird excreta. Bird excreta could act as a potential carrier in the spread of pathogenic yeasts such as *Cryptococcus neoformans* into the environment which get transmitted to children and elderly as well as immunocompromised patients (Soltani *et al* 2013) ^[18].

Table 3: Occurrence of *Cryptococcus neoformans* in excreta of different bird species

Bird species		<i>Cryptococcus neoformans</i>
Blue rock pigeon	Wet (n=5)	+
	Dry (n=2)	+
*Rose ringed parakeet (n=1)		-
*Common myna (n=1)		-
*House crow (n=5)		+
*Cattle egret (n=2)		-
*Red wattled lapwing (n=1)		-

* tested with wet excreta

+ refers to detected

-refers to undetected

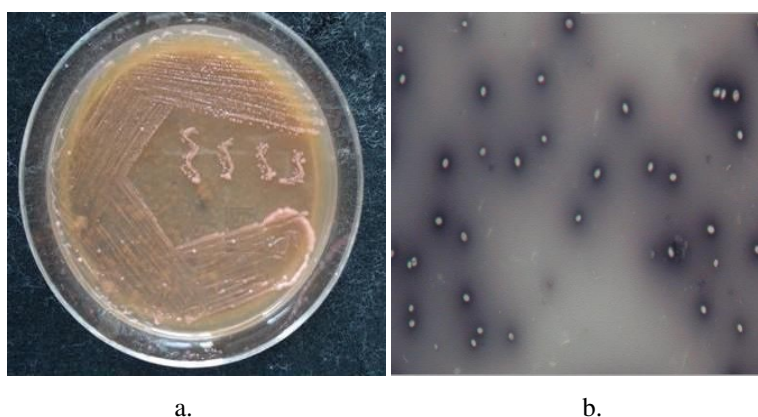


Fig 5: a. *Cryptococcus neoformans* colonies isolated from excreta of birds
b. Capsule of *Cryptococcus neoformans* on India ink preparation (Magnification 400 X)

Thus from the fungal analysis of excreta of different bird species, it can be concluded that excreta of birds in agroecosystems harbor a wide range of pathogenic moulds and infectious yeasts. As these birds are free living, so spread of infectious agents occur through faecal contamination of drinking water sources, pastures and agricultural crops thus enabling the direct transfer of infectious agents to take place which may be a source of infection to human beings as well as may be harmful to birds themselves.

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