Isolation of Cryptococcus neoformans from pigeon excreta in Qazvin

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Abstract: Background: Cryptococcus neoformans is a major pathogen in patient's immunocompromised, ubiquitous fungi that can live free from dust, bird droppings and plant materials are separated. Objective: purpose of this study was isolation of Cryptococcus neoformans from pigeon excreta in Qazvin. Methods: The total of 50 pigeon excreta were collected and cultured on the brain heart infusion agar and Saboured dextrose agar with Chloramphenicol media, to isolate Cryptococcus neoformans at the 25 degrees centigrade and 37 degrees centigrade. Identification was performed by direct examination and by means of the urease test and Carbohydrate assimilation. Finding: From 50 pigeon excreta 2 cases (4%) Cryptococcus neoformans were detected. Besides the Cryptococcus neoformans were isolated 10 species of Cryptococcus Candida unigattulatus 4 cases (5.72%), Candida laurentii 3 cases (4.28%), Candida albidus 2 cases (2.86%) and Candida humicola 1 cases (1.43). Conclusion: Immunosuppressed individuals, especially HIV infected persons (AIDS patients) should avoid contact with pigeons and AIDS patients should avoid contact with pigeons and pet birds.
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1. Introduction

Cryptococcus neoformans is opportunistic and basidiomycete yeast that cause meningitis in immunocompromised patients. People having cryptococcus are usually AIDS patients. Origin of Cryptococcus is mostly pigeon feces, but it has been isolated from feces of other birds like parrots and sparrows too.

Pet bird's excreta and those around us can be a reservoir for this pathogen. Causative agents of Cryptococcus neoformans are neoformans and gattii varieties, and infections spread to human through environmental sources. Cryptococcal fungal infection is transmitted by soil and air contaminated, therefore immune-deficient people and people who are HIV-infected should avoid of contact with feces of tamed birds (Lugarini, 2008).

Cryptococcus neoformans causes cryptococcus probably rarely is at the people with natural immunity (Blaschke-Hellmessen, 2000).

Infections with gattii variety mostly have been reported from Australia, Brazil, Hawaii, Southeastern California, Mexico, Thailand, Vietnam, Nepal and central African countries, or generally speaking, tropical and sub-tropical areas while neoformans variety have been reported from Denmark, Belgium, France, Germany, Netherland, Italy, Switzerland, and Japan(Kwon-Chung and Bennett, 1984).

In 2005, Gugnani has reported segregation gattii of Clemens flowers and eucalyptus trees in India, but the variety neoformans from pigeon excreta have been isolated (Gugnani, 2005). Tame pigeons and wild pigeon healthy, can be used as both carrier types neoformans function (Blaschke-Hellmessen, 2000). 5-10% of patients with advanced HIV infection with disseminated cryptococcus have been reported. This infection is the most common lethal fungal infection in these population (Mitchell, 1995), and almost in 40% of cases, this is the first presenting sign of getting infected with AIDS (Mitchell, 1995; Powderly, 1995). Always CD4 fraction of T lymphocytes are less than 100 in 1cc, but usually it is less than 50 at the time of diagnosis (Shankar, 2007). Cryptococcus neoformans are an encapsulated yeast and causative agent of cryptococcosis in man and animal. The environmental source of Cryptococcus neoformans is pigeon's feces and it is a cause of death in immune deficient patients (Shankar, 2007).

Levits, (1991) stated that Cryptococcus neoformans rarely produces cryptococcosis in people with normal immunity and the infection is mostly limited to AIDS patients (Levitz, 1991).

Faggi, (1993) mentioned that domestic animals like dogs and cats also are infected with cryptococcosis. He has reported of cryptococcosis infection at the nervous system in three dogs and two cats (Faggi, 1993).

Mitchell, conducted a study that prognosis of cryptococcosis with gattii variety of Cryptococcus neoformans is worse than neoformans variety in human. Gattii variety usually affects on the people residing in rural area or those having occupation in

rural areas. He studied 118 cases of nervous system cryptococcosis from 1985 to 1992 and stated that cryptococcosis with gattii variety is several times more prevalent than neoformans variety in Australia (Mitchell, 1995).

Bava also stated in 1997 that AIDS is a predisposing factor for acquisition of cryptococcosis. He noted that men were at a higher risk of getting infection, and this was due to higher prevalence of AIDS in men during the years 1981 through 1993 (Bava, 1997).

Arteaga, (1998) has reported 211 cases of autopsies of patients with AIDS that 29% of them had nervous system cryptococcosis, 9.6% histoplasmosis and 3.2% pulmonary aspergillosis(Arteaga Hernandez, 1998).

In the year 2000, Blaschke stated that immune deficient people and those having AIDS or HIV should avoid contact with feces of domestic birds and pigeons, since they may have Cryptococcus neoformans and cause infection of these patients with cryptococcosis. He described that wild and tamed birds and pigeons that are healthy carriers of Cryptococcus neoformans and this yeast is mixed with feces and produces infection through inhalation of contaminated soil by susceptible people (Blaschke-Hellmessen, 2000).

In 2001, Pitisuttithum stated that high doses of Amphotericin B were not as effective as it was thought in preventing mortality of AIDS patients having cryptococcosis. He provided this conclusion after one year of studying on 106 AIDS patients with cryptococcal meningitis being treated with Amphotericin B and regular culturing of spinal fluids of these patients and evaluation of Cryptococcus yeasts in spinal fluid samples (Pitisuttithum, 2001).

In the year 2002, Iyer stated that PCR can be used as a rapid tool for diagnosing cryptococcal meningitis. All samples becoming positive in culture were confirmed as positive by PCR too (Iyer, 2002).

In the year 2003, Malik presented a report of cryptococcosis of Australian parrots and stated that cryptococcosis in parrots involves nasal cavity, upper respiratory tract, beak, sinuses and facial surfaces. Causative agent of four parrots was gattii variety of Cryptococcus neoformans. It seems that sitting of parrots on eucalyptus tree containing gattii variety of Cryptococcus neoformans yeast has acted as a predisposing factor for getting cryptococcosis. Malik also reported two cases of infection with grubii variety of Cryptococcus neoformans in parrots due to skin trauma from America (Malik, 2003).

In the year 2006, Dharmshale reported wide spread skin involvement with Cryptococcus neoformans in AIDS patients having cryptococcosis. He stated that skin infection with this fungus is seen in 15% of AIDS patients having disseminated cryptococcosis (Dharmshale, 2006).

In the year 2006, Seo presented a report of prostatitis with Cryptococcus neoformans. He reported a case of prostatitis with Cryptococcus neoformans in an immune deficient alcoholic patient having cirrhosis, where the diagnosis had been established with sonography and biopsy (Seo, 2006).

In the year 2006, Andreola reported a case of ophthalmic choroiditis involvement in a 27 year old woman having HIV with disseminated cryptococcosis infection. In histological examination of the eyes after her death, presence of Cryptococcus neoformans yeast was established. Detecting this condition by the ophthalmologist can influence treatment and prognosis of the disease (Andreola, 2006).

In the year 2007, Umemura reported a case of cryptococcal meningoencephalitis in a male AIDS patient after autopsy. In autopsy of his brain, multiple cysts caused from free from Cryptococcus fungus in these patients, physicians should apply a greater care in diagnosis (Umemura, 2007).

In the year 2007, Shankar spoke of having conditions like AIDS, cancer, organ transplant, immune deficiency and … as a way of getting infection with Cryptococcus (Shankar, 2007).

In the year 2008, Yoshida put forth measurement of glucuronoxy lomannan, a capsule structural antigen of Cryptococcus fungus for diagnosing cryptococcosis, and suggested using drugs like fluconazol, itraconazol, and Amphotericin B together with flucytosin (Yoshida, 2008).

Cabanes, (1995) stated that cryptococcosis in domestic animals is an uncommon event and it is seen as a sporadic infection. These infections often take place with neoformans variety, but infections with gattii variety are also seen in different species of animals. Among domestic animals, the highest prevalence of cryptococcosis belongs to cats (Cabañes, 1995).

In the year 2008, Kwon-Chung stated that most isolates of grubii variety of Cryptococcus neoformans (serotype A) are not lethal for mice. Most cryptococcosis in man is caused from grubii variety of Cryptococcus neoformans, which is ubiquitous in the nature and spreads with pigeon's feces. He used mice nasal injection model of clinical lethality for testing pathologic strength of environmental strains of serotype A Cryptococcus neoformans and from eleven strains tested, only one strain produced illness in mice in 60 days after injection (Chen, 2008).

In the year 2010, Liaw evaluated 100 cases of cryptococcal isolates with serotype, molecular and pathologic factors methods. Eight isolates were from

pigeons feces and the remaining was clinical. 99 isolates of the whole 100 were grubii variety of Cryptococcus neoformans, and the other one was gattii variety of Cryptococcus neoformans, serotype B. all isolates were tested for production of urease, phospholipase, capsule and melanin, and the amounts of production were different in isolates. Melanin production has a direct influence in meaningful protection of Cryptococcus against being killed by Amphotericin B in isolates (Liaw, 2010). Since Cryptococcus neoformans is found in places where pigeons haunt, haunting of pigeons in urban places where immune deficient patients are kept can be dangerous for their health.

2. Material and Methods

This is an analytical descriptive study carried out in Qazvin from 2009 to 2010. The study population consists of pigeons feces at the Qazvin. Qazvin was divided into 25 areas in accordance with postal map of the city. During this period, two saplings were planted in each area. Sampling was classified as a Census study was done while we were trying to check all the points. In each postal area, from houses where pigeons were kept, some feces of pigeon were taken from cages. Feces from under roofs of houses, holy shrines, and pigeon selling shops were also collected. In later stages, suspensions were made from the collected feces and some antibiotics like penicillin or streptomycin were added to them in order to reduce bacterial population. Before preparing suspensions, samples were placed in nylon bags and mixed thoroughly, and then, one gram of it was put in a tube and 9 cc of physiologic serum was added to it. Then, tubes were shaken for 5 minutes and left stationary for 30 minutes, and then, cultures were made from the supernatant fluid part, with a sterile device and in Sabouraud dextrose Agar containing chloramphenicol. Two culture plates were prepared from each sample, one to be taken to a 37 degree incubator and the other to be kept at 25 degrees centigrade. After 48 hours, routine mycological examinations like observing the fungus with Indian ink, using urea culture media, corn meal agar , chrome culture media and sugar kit like api 20C- Aux were carried out on colonies appeared. For transferring the fungus to sugar media, first, the fungus was transferred to a Brain Heart Infusion Agar (BHI) media to clarify the fungus off from sugar. Then, a suspension with turbidity of 2 Mc Farland was prepared from the fungal colonies grown in this suspension and the instructions of the related sugar kit were followed. For injecting to the lab animal (souris), a sample, approved to be Cryptococcus neoformans via all tests carried out, was taken and turned into a suspension of one million spore in each ml from its new culture, and injected in amount of 0.3 ml through caudal vein, and 0.8 ml through peritoneum. Brain, liver and spleen tissues were taken from dead mice and stained with Indian ink, gimsa, and methylen blue. Preparing blocks and staining the slices with hematoxilen eosine were also done. For preparing suspensions to be injected to mice, counting spores on neobar slides was also used. Feces of parrots, love bird, canary and poultry were also cultured in Sabouraud dextrose Agar for finding Cryptococcus. For analyzing data, descriptive statistics methods were exploited.

3. Results

This study was done in 6 months (February through August 2010) in Qazvin, Iran. In this study, 70 types of colonies of different yeasts and saprophytes were isolated from 50 samples of pigeon feces (table 1).

Colonies suspected to be Cryptococcus produced a brownish color after being transferred to chrome agar media, and produced blastoconidia after being transferred to corn meal agar media. Injection of 10 to power 6 Cryptococcus neoformans in ml suspension to Souris mice led to death after 5 to 12 days. In evaluating the brain, liver and spleen of these mice, encapsulated yeast cells were seen with Indian ink. Urea test after 10 minutes to 48 hours to was positive. Cryptococcus neoformans isolated from pigeons feces grew in Sabouraud dextrose Agar at both temperatures of 25 and 37 degrees centigrade.

Table 1. Frequency of isolated subtypes

Fungus type	Number of isolates	percent
Cryptococcus ioni gotulatus	4	5.72
Cryptococcus larneti	3	4.28
Cryptococcus neoformans	2	2.86
Cryptococcus albidus	2	2.86
Cryptococcus homicula	1	1.43
Candida crusei	6	8.57
Candida albicans	4	5.72
Rhodotorula glotinis	2	2.86
Geotrichum capitatum	3	4.28
Trichosporon asahi	2	2.86
Aspergillus spp.	15	21.43
Rhizopus spp.	12	17.14
Mucor spp.	6	8.57
Penicillium spp.	5	7.14
Fusarium spp.	3	4.28
Total	70	100

Culture taken from feces of domestic birds like canary, love bird, parrot and poultry were all negative. The result of sugar absorption with api 20 c AUX kits was 12 instances of different Cryptococcus species from the whole 50 pigeon feces samples (24%) and isolation of two Cryptococcus neoformans yeasts (4%). Isolated cryptococcuses were obtained

from pigeon feces collected from dark and shadow places with no access to sun light. In samples collected from different geographical regions of Qazvin, meaning north, south, east, west and center, no difference was observed from view point of different Cryptococcus species. From the two cases of Cryptococcus neoformans, one was obtained from east and the other from west. No difference was observed from viewpoint of presence of Cryptococcus divided by place of collection including cage floor, under roof, or on the walls. Saprophyte fungi like Aspergillus, Mucor, Rhizopus, and Pencillium, and yeasts like Rhodotorula, Trichosporon, Geotrichum and Candida and bacteria also appeared on cultures of pigeon feces.

4. Discussions

In this study, two isolates of Cryptococcus neoformans were obtained from samples of pigeon feces of Qazvin.

Chung (1984) has stated that more than 85 percent of cryptococcuses isolated in Argentina, Canada, England, America (except south –east California) belong to neoformans variety of Cryptococcus neoformans and the remaining 15 percent belong to gattii variety of Cryptococcus neoformans. High prevalence of gattii variety of Cryptococcus neoformans (35 to 100 percent) is seen in Australia, Brazil, and south –east California, Mexico, Paraguay, Vietnam, Nepal, and central African countries. He states that gattii variety of Cryptococcus neoformans is prevalent only in tropical and subtropical areas (Kwon-Chung and Bennett, 1984). Serotype studies in these areas showed that 70 percent of isolates of neoformans variety of Cryptococcus neoformans were of a serotype, and from the two serotypes of gattii variety of Cryptococcus neoformans, B serotype was 4.5 times more than C serotype (Chen, 2008).

In 1993, Li isolated Cryptococcus neoformans from pigeon feces in China and stated that 78 percent of isolates were of a serotype and 22 percent of AD serotype. He found only neoformans variety of Cryptococcus neoformans from pigeon feces in China, although gattii variety of Cryptococcus neoformans has also been isolated from clinical samples in China and this fungus has a special place in the nature and is specific to tropical and subtropical areas (Li, 1993).

In 1998, Yildriran isolated 29 cases of Cryptococcus neoformans from 634 samples pigeon feces in Turkey. In this isolation, air humidity of air and being away from sun light were influential factors (Yildiran, 1998).

In 2000, Kielstein proved presence of neoformans variety of Cryptococcus neoformans in feces of pigeons and domestic birds through PCR method. 50 percent of isolates belonged to Cryptococcus albidus (Kielstein, 2000).

In 2002, Horta reported isolation of 17 clinical and 10 environmental Cryptococcus neoformans cases from pigeon feces in Rio de Janeiro state of Brazil (Horta, 2002).

Tay (2005) also reported 20 isolates of Cryptococcus neoformans from feces of zoo birds, bird selling shops and public places of Clang city in Malaysia. All isolated strains of Cryptococcus neoformans were of a serotype and identified as grubii variety of Cryptococcus neoformans. All isolates were sensitive to Amphotericin B, Itraconazol (Tay, 2005).

Gugnani (2005) reported isolation of two varieties of gattii and grubii of Cryptococcus neoformans from eucalyptus trees in India. He collected 233 samples from eucalyptus tree parts including flowers, barks, and leaves of the tree. Two cases of Cryptococcus gattii were isolated from flowers and two other grubii varieties of Cryptococcus neoformans from barks of the tree (Gugnani, 2005).

In 2005, Rosario isolated Cryptococcus neoformans from cloak of pigeons in Spain. He prepared 331 samples from cloak of pigeons with a swab, of which, 26 cases had Cryptococcus spp (7, 58%), 11 (3.32%) had Cryptococcus Uniguttulatus, 6 (1.81%) had Cryptococcus Laurentii, 6 (1.81%) had neoformans variety of Cryptococcus neoformans, and 3 (0.91%) had Cryptococcus albidus. The results showed that pigeons act as a reservoir for cryptococcosis and as a carrier for neoformans variety of Cryptococcus neoformans (Rosario, 2005).

In the year 2005, Chee reported isolation of serotype a grubii variety of Cryptococcus neoformans from feces of pigeons in Seoul, Korea. 72 samples of pigeon feces sere collected from 25 points of Seoul city, 17 cases of which from 8 points contained Cryptococcus neoformans. All isolates were obtained from dry feces of pigeons, and all went under identification and serotyping through DNA fingerprinting, where all turned to be A serotype grubii variety of Cryptococcus neoformans (Chee, 2005).

Cermeno(2006) isolated Cryptococcus neoformans from doves in BolivGorganar province of Venezuela (Cermeño, 2006).

Duncan (2006) obtained Cryptococcus gattii from nasal culture of a gray squirrel in Vancouver Canada, and stated that wild animals of Vancouver can become a reservoir for this fungus, like domestic animals of this region (Duncan, 2006).

In the year 2006, Abegg from Brazil, reported isolation of Cryptococcus neoformans and

Cryptococcus gattii from bird's garden of Rio de Janeiro. He obtained 32 isolates belonging to a serotype grubii variety and 5 isolates belonging to B serotype Cryptococcus gattii. All isolates were evaluated for virulence factors and quantitative production of urease, and the urease amount of gattii variety was similar to the isolates obtained from clinical cases (Abegg, 2006).

Rosario (2008) reported from Spain that pigeons were not the only reservoir of Cryptococcus, and other birds could act as reservoir for it too (Rosario, 2008).

In 2008, Lugarini from Brazil reported isolation of Cryptococcus neoformans from feces of parrots and sparrows. He stated that feces of domestic birds and those around us, act as reservoir for Cryptococcus neoformans (Lugarini, 2008).

In 2008, Baltazar from Spritosanto state of Brazil reported an isolation of Cryptococcus gattii from trees of the region. In addition to Cryptococcus gattii, he isolated Cryptococcus laronti and Cryptococcus neoformans from woods trees too. He stated that environment is a source of infection with this fungus (Baltazar Lde, 2008).

In 2010, Liaw reported isolation of Cryptococcus neoformans from feces of pigeons and stated that 99 percent of Cryptococcus neoformans isolates he evaluated in his study were a serotype of grubii variety (Liaw, 2010).

In 2010, Centers from America reported isolation of Cryptococcus gattii from woods trees of four states of Iowa, Oregon, Washington, and Idaho. He states that Cryptococcus neoformans mostly inflicts people having HIV, while Cryptococcus gattii mostly contaminates people not having HIV in tropical and subtropical areas (Centers for Disease Control and Prevention, 2010).

In 2009, Souza isolated Cryptococcus gattii; serotype B from eucalyptus trees (Souza, 2010).

We used Sabouraud dextrose Agar for culturing Cryptococcus, and Menezes (2002) used the same as culture media. We used direct observation method with Indian ink for seeing capsules of Cryptococcus fungus grown on culture media, again, as how Menezes had done (Menezes, 2002). For identification of species, urease test and absorption of different sugars were used, like what Li had done (Li, 1993). And finally, we used PCR method for identification of isolates, and Kielstein used the same PCR method for identification of isolates (Kielstein, 2000). Since Cryptococcus neoformans is a basidiomycete opportunistic yeast, mostly infeccting immune deficient and specially AIDS patient (Lugarini, 2008), and also since this yeast was found in feces of pigeons of Qazvin city, it is necessary that more attention be paid to possible infliction of AIDS patients of Qazvin with this fungus.

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