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Isolation and molecular identification of pathogenic cryptococcus neoformans from pigeon dropping and human sputum

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***Abstract*---**This study is designed to isolate and molecular identification of C. neoformans, C. neoformans is pathogenic yeast and effect immunocompromised and immunocompetent. Methods: collect 50 samples from pigeon dropping and 50 samples from pigeon fanciers (sputum). The collection time was extended from November 2021 to February 2022, then culture at SDA, BSA, Cryptococcus Differential agar, esculin agar, Eucalyptus leaves agar media and Brain heart infusion agar with methyldopa, biochemical test including urease test and methyldopa, and then confirm identification by molecular identification by PCR technique sequencing and genetic analysis. The results showed that 3 swaps taken from sputum of human included cryptococcus neoformans and 6 swaps from bird dropping included cryptococcus neoformans. This study indicated that the virulence and normal habitat of cryptococcus neoformans. These results indicate that there may be a risk for human exposure to cryptococcal disease due to inhalation of basidiospores from dropping of pigeon in pigeon houses

***Keywords*---**isolation, molecular identification, pathogenic cryptococcus neoformans, pigeon dropping, human sputum.

Introduction

Fungi responsible and causing mycosis infectious are responsible for (1) superficial and cutaneous mycosis (2) subcutaneous mycosis (3) deep systemic mycosis caused by the opportunistic yeasts or filamentous fungi and this is estimated to be Infections with Cryptococcosis, Candidiasis, and Aspergillosis

have been reported in the United States, Europe, Africa, and other nations. (De Hoog *et al.*, 2005). Cryptococcus is an invasive fungus that causes cryptococcosis, a disease that affects persons with weakened immune systems but is uncommon in healthy people. Two Cryptococcus species are usually connected with infections in humans: Cryptococcus neoformans and Cryptococcus gattii. In certain regions of the world, the organism is very prevalent. Immune suppression is the essential mechanism in the causation of cryptococcal illness. Diseases like chronic liver disease, chronic renal disease, AIDS, patients who undergo organ transplantation and prolonged use of steroids are commonly with relation to the development of cryptococcal disease (*Lin, et al., 2015*).

Cryptococcus neoformans is a non-fermenting fungus cell that is enclosed and spherical. (Okagaki *et al.*, 2010). C. neoformans is unique among pathogenic fungi in that it has a mucinous capsule. At 37°C, basidiospores with a diameter of 1.8 to 3.0 m may produce yeast cells, whereas at 24°C, they can produce dikaryotic hyphae (Karkowska-Kuleta *et al.*, 2009). C. neoformans causes deadly meningitis in immunosuppressed individuals (Cogliati, 2013). The yeast form is usually found in tissues. Apart from an asexual life cycle, C. neoformans possesses a bipolar mating cycle that includes two types of mating, MATa and MATα, with the last being the most isolated from the environment and hosts (Cogliati, 2013). Cryptococcus neoformans is isolated from pigeon and other bird faeces more frequently than from other environmental and veterinary (cow, dog, horse, cat, sheep) sources, according to an environmental assessment (Cogliati, 2013).

Material and Method

Collect of human (sputum)

Collect 50 sputum samples. The collection time was extended from November 2021 to February 2022, from pigeon fanciers in different ages, and then transmitted under aseptic conditions to the college laboratory, all these samples were cultured in the laboratory where they were collected and directly after collection inoculate them on sabouraud dextrose agar with chloramphenicol. Sputum were streaked simply with a loop on sda media (Staib *et al.*, 1987). If the growth appeared diagnosis by doing smear on a slide and staining it with India ink and lactophenol cotton blue in addition to the urease test, and confirmed by culturing on the bird seed agar media and cryptococcus differential agar media and other identification tests.

Collect of pigeon dropping

A total of 50 Samples were collected in sterile plastic bags from bird dropping in pigeon houses (Bird Market in New Baghdad, Muhammad Ali's Hill and Shorja yarn market). A portion of each sterile plastic bag's droppings (about 20-30 g) was aseptically collected, weighed, and transferred to Erlenmeyer vials holding a 0.9 percent saline solution with 200 mg/L chloramphenicol, resulting in a 1:10 dilution (w/v). Shaking was used to homogenize the material, which was then set aside for 30 minutes. On Sabouraud's dextrose agar medium, aliquots of 0.5 ml supernatant were streaked (Zarrin *et al.*, 2010). If the growth appeared diagnosis

by doing smear on a slide and staining it with India ink and lactophenol cotton blue in addition to the urease test, and confirmed by culturing on the bird seed agar media and cryptococcus differential agar media and other identification tests. The collection time was extended from November 2021 to February 2022.

Preparation of media

Sabouraud dextrose agar (SDA), Cryptococcus Differential Agar and Bile Esculin agar was prepared manufacturer's instructions.

Bird seed agar

This media was prepared by added 50.0g Guizotia abyssinica seed grinding by mortar and a pestle, then add 1.0L of distilled water, Boil for 30 min, Filter through sterilized gauze and filter paper, Then Add 20g agar to seed filtrate, Mix thoroughly and heat until boiling, Distribute into flasks, Autoclave for 15 min at 15 psi pressure-121°C. (Atlas & Snyder, 2013).

Eucalyptus leaves agar media

The 2g leaves of eucalyptus were rinsed in sterile distilled water and were pulverized with a sterile mortar and a pestle then immersed in 20ml sterile saline solution supplemented with chloramphenicol and homogenized with ultrahomogenization (vortex) for 4 min, then I leave it for two days, then filter it by filter paper to flask and add 2g agar for 100 ml and heated then sterilize by autoclaving at 121°C under 15 pounds for 15 minutes (Refai *et al.*, 2005; Elhariri *et al.*, 2016).

Brain and heart infusion agar with methyldopa

The brain heart infusion agar was prepared by dissolving 52 grams of brain heart infusion agar powder in one litter of distilled water. The solution was mixed thoroughly and heated with frequent agitation then sterilize by autoclaving at 121°C under 15 pounds for 15 minutes, the sterile media was cooled to about 50°C then added with methyldopa from a pharmaceutical tablet (one Pill 250mg for 250ml) (Menezes *et al.*, 2011).

Staining
India ink

This test was performed by placing a drop of distilled water or normal saline on the slide, mixing it with the yeast growth colony, then adding a drop of India ink after drying the slide, covering the slide, and examining it under a 40 X lens. This test was performed to detect the capsule that surrounds a cell the Cryptococcus (Baron & Finegold, 1990).

Molecular identification
DNA extraction

Colonies from cultures were inoculated into 1.5 mL Eppendorf tubes holding 0.5 mL Sabouraud Dextrose broth supplemented with chloramphenicol and incubated overnight in an orbital shaker at 150 rpm at 30°C. 24 hour later, centrifugation of the fungal suspensions have been occurring for ten min at 5,000 rpm, and freeze the pellet for 1 hour at minus 20°C before being incubated at 65°C for one hour in 0.5 ml extraction buffer holding 50 mM Tris-HCl, 50 mM EDTA, 3 percent sodium dodecyl sulfate, and 1 percent 2-mercaptoethanol. Finally, the lysate was extracted in a 25:24:1 ratio with phenol, chloroform, and isoamyl alcohol (v/v). 65 liters of 3 M sodium acetate and 75 liters of 1M sodium chloride were poured into this, and the mixture was incubated for 30 minutes at 4°C. Isopropanol precipitation was used to recover DNA, which was then washed with ethanol 70% (v/v). In a UV-VIS Spectrophotometer, the DNA conc. was examined at 260 nm and kept at minus 20°C until future use (Tendulkar *et al.*, 2003; Mseddi *et al.*, 2011; Moťková, & Vytřasová, 2011).

PCRamplification

for characterization of *Cryptococcus* species, The regions of the primers employed for amplification targeted ITS1 and ITS4, with predicted fragment lengths of 500 650 bp. Table 1 contains information about the primers .Denaturation for one minute at 940C, primer annealing for one minute at 550C, chain extension for one minute at 720C, and final extension for seven minute at 720C were followed by 35 cycles of denaturation for 1 minute at 940C. The PCR products were resolved in a 1.5 percent agarose gel, electrophoresed, and visualized using a UV gel documentation system after amplification. For subsequent investigation, the amplicons were kept at -200C (Mitchell *et al.*, 1994; Lau *et al.*, 2007; Lusia Leal *et al.*, 2008; Sidrim *et al.*, 2010).

The primers used in the interaction

Table 1: The specific primer of gene *ITS 4*

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5′- TCCGTAGGTGAACCTGCGG -3′	60.3	50 %	500-650 base pair
Reverse	5′ TCCTCCGCTTATTGATATGC-3′	57.8	41 %	

Result and Discussion

According to primary isolation that depended on (direct examination) by using wet mount smear staining with India ink characterization of Cryptococcus neoformans growth on SDA, bird seed agar, cryptococcus differential agar, Eucalyptus leaves agar media, Brain heart infusion agar with methyldopa and urease test,. 6 (12%) sputum samples out of 50 isolations and 9 (18%) pigeon dropping out of 50 samples was diagnosed as cryptococcus spp.

Table 2: Result of Primary isolation in lab

Sources of sample	No. of samples	Primary isolation in lab	
		No. of +ve	%
Sputum of human	50	6	12
Dropping of pigeon	50	9	18

The yeast can be grown on the sabouraud dextrose agar and develops white, creamy, glistening and convex glistening colonies within 2-3 days of incubation, for C.neoformans the mucoid appearance of colonies is caused by the cells production of a polysaccharide capsule, these findings were in agreement with (Khanal *et al.*, 2002).

Microscopic with India ink for C. neoformans capsule

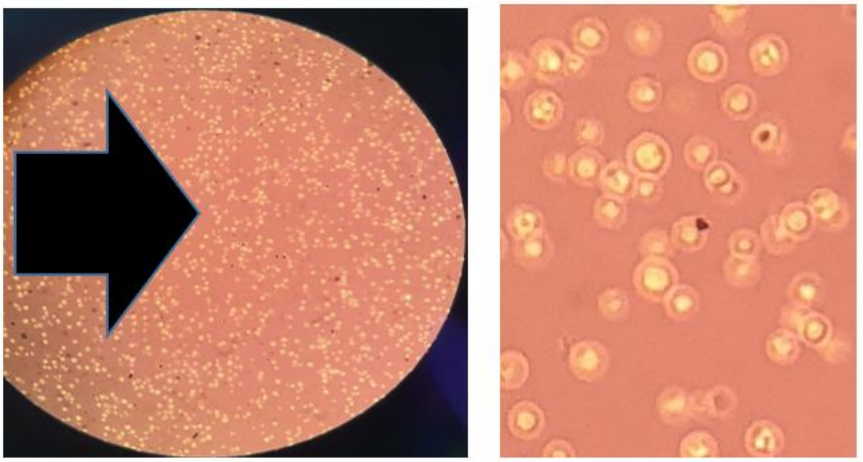

Figure 1: C. neoformans capsule stain with india ink under 40x lens

Melanin production

Bird seed agar

Individual colonies showed growth and pigment colonies formation after 2–3 days of incubation at 37°C temperatures. After a two-day incubation period, and this is agree with (Pham *et al.*, 2014).

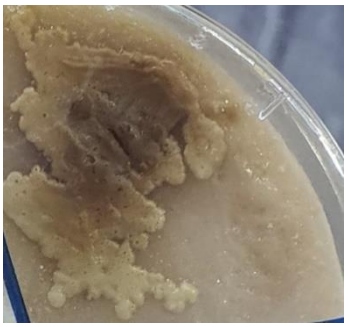

Figure 2: C. neoformans give brown color in bird seed agar media after 2–3 days of incubation at 37°C temperatures

Cryptococcus Differential Agar

C. neoformans appear as Light blue, dry colony so that Pigmentation is not visible on the first day of growth but becomes obvious after five days of incubation, and its intensity increases gradually over time after 2-3 weeks which were showed by (Chaskes *et al.*, 2008).

Figure 3: growth of C. neoformans on Cryptococcus Differential Agar after 10 days of incubation at 37°C temperatures

Esculin agar

All other yeasts generated no pigment or were light yellow, except Cryptococcus spp., which formed a brown-black pigment on the esculin agar. Beta-glucose-6,7-dihydroxycoumarin is the chemical name for esculin. Because the esculin molecule's component 6,7-dihydroxycoumarin was transformed into a melanin-like pigment, which were produced by C. neoformans isolates and this is agree with (Almeida *et al.*, 2015)

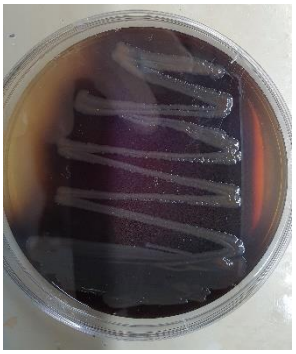

Figure 4: Cryptococcus spp. on esculin agar after 1 days of incubation at 37°C temperatures

Brain heart infusion agar with methyldopa

Colonies of melanin-generating species of Cryptococcus display a show of colors ranging from brown to black when developed in agar media, so Brain heart infusion agar with methyldopa, which was as revealed by effective as a melanin-producing substrate (Menezes *et al.*, 2011).

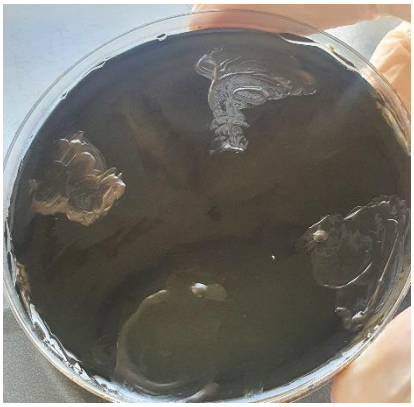

Figure 5: Cryptococcus spp. on Brain heart infusion agar with methyldopa after 2 days of incubation at 37°C temperatures

Eucalyptus leaves agar media

Cryptococcus species that produce melanin display a show of color with different brown spectrum in their colonies, on Eucalyptus leaves agar medium, so C. neoformans isolates produces a brown color and this is agree with (Elhariri *et al.*, 2016).

Figure 6: Cryptococcus spp. on Eucalyptus leaves agar media after 2 days of incubation at 37°C temperatures

Urease test

The capacity of the isolated strains of yeast to utilize urea and liberate ammonia was demonstrated by the ability of the yeast to generate urease enzyme and a change in the color of the medium from yellow to pink or light purple after 48 h of incubation at 25 degrees Celsius which agree with the finding of (Li & Wu, 1992).

Figure 7: Control and positive urease test for Cryptococcus spp. after 2 days of incubation at 37°C temperatures

Results of sequencing and genetic analysis

After sequencing and genetic analysis showed 3 (50%) sputum samples out of 6 isolations and 6 (66.6%) pigeon dropping out of 9 samples.

Table 3: Results of sequencing and genetic analysis

Sources of sample	No. of samples	Results of sequencing and genetic analysis	
		No. of +ve	%
Sputum of human	6	3	50
Dropping of pigeon	9	6	66.6

The sequencing reactions indicated the exact identity of these infectious sequences after executing NCBI blast n for their PCR amplicons (Zhang *et al.*, 2000). The NCBI BLASTn engine found 99 percent sequence similarity between the sequenced samples and the targeted reference target sequences for the 500-650 bp amplicons. The precise locations and other properties of the obtained PCR fragments were determined by comparing the nucleic acid sequences of these

investigated samples with the returned nucleic acid sequences. (JQ794497.1, MG461667.1 and NG 064879.1). The NCBI server was used to establish the total length of the targeted locus, and the start and end sites of the targeted locus were confirmed using the most comparable fungal target.

The features of ITS4 sequences were emphasized after situating the 500-650 bp amplicons' sequences inside the yeast' genomic sequences, and the overall length of the amplified fragments was also calculated. The length and positions of the 500_650 bp PCR amplicons that was utilized to amplify a portion of the ribosomal sequences within Cryptococcus neoformans genomic sequences (Gen Bank acc. no. JQ794497.1, MG461667.1 and NG_064879.1) interestingly, the alignment findings of the 500_650bp samples revealed the existence of more nucleic acid variations which were represented by much nucleic acid substitutions compared to the most closely related referencing nucleic acid sequences. These nucleic acid substitutions were found to be distributed specifically in the investigated fungal samples. Sequencing reactions revealed that much variant in pigeon dropping samples which is represented by one samples have one variant (Transvertion A-C 204), three samples have two variant (Transvertion T-G 38, Transition G-A 40), (Transition T-C 74, Transvertion T-A 82) and (Transvertion A-C 204, Transition A-T 378) and two samples have three variant (two Transition G-A 36, A-G 40 and Transvertion T-G 38), while in human sputum samples which is represented by two samples have two variant (Transvertion A-C 1001, Transvertion T-A 1353), (Transvertion T-A 1353, Transition C-T 1420) and one samples have three variant (Transvertion A-C 1001, Transvertion T-A 1353, Transition C-T 1420)

Observed variants of (A-C 204, T-G 38, G-A 40,T-C 74, T-A 82,A-C 204, A-T 378, G-A 36, A-G 40, T-G 38, A-C 1001, T-A 1353, T-A 1353, C-T 1420 A-C 1001, T-A 1353, C-T 1420) were not detected in corresponding of reference sequences. The confirmation of incredibly wide variations, the analyzed samples' sequencing phylogenetic, as well as their thorough annotations, were validated and documented. Their sequences' phylogenetic were displayed in order of their locations in the PCR amplicons. Each variant's presence was validated in its original chromatogram, as was the lack of any probable technical mistake.

To provide a phylogenetic comprehension of the real distances between the currently studied samples and their respective sequences, a thorough phylogenetic tree was constructed in this work based on nucleic acid differences found in the amplified 500_650bp ribosomal sequencing amplicons. This phylogenetic tree included the currently analyzed sample as well as other relative C. neoformans nucleic acid sequences.

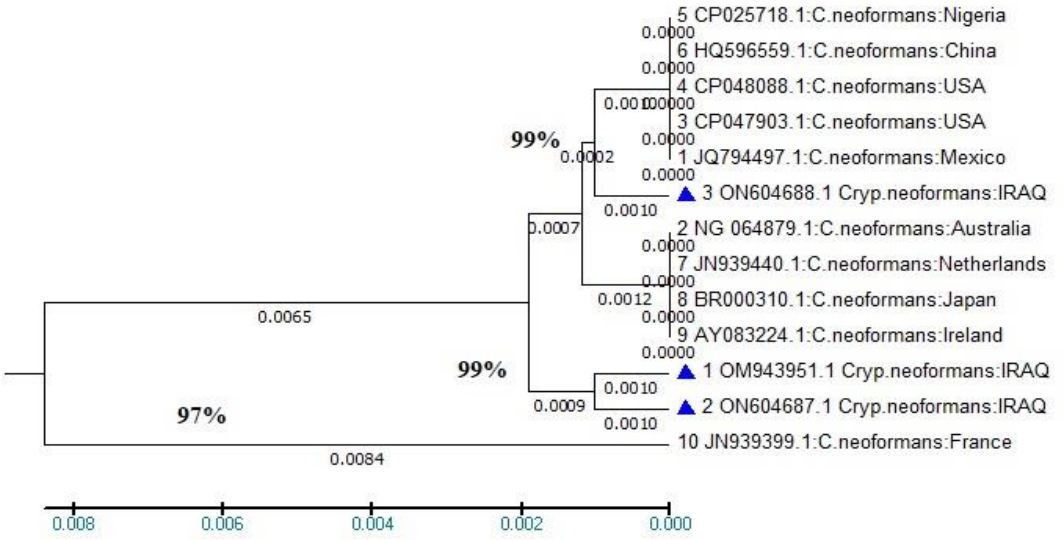

Figure 8: The phylogenetic tree for samples of human sputum have 10

Observation through phylogenetic tree the strains numbers OM943951.1 and ON604687.1 were confirmed by other neighboring sequences since it was found that GenBank isolates under the accession numbers AY083224.1, BR000310.1 which was deposited from Ireland and Japan matching is get 99%. While the strains numbers ON604688.1 1 were confirmed by other neighboring sequences since it was found that GenBank isolates under the accession numbers CP047903.1 and JQ794497.1 which was deposited from USA and Mexico.

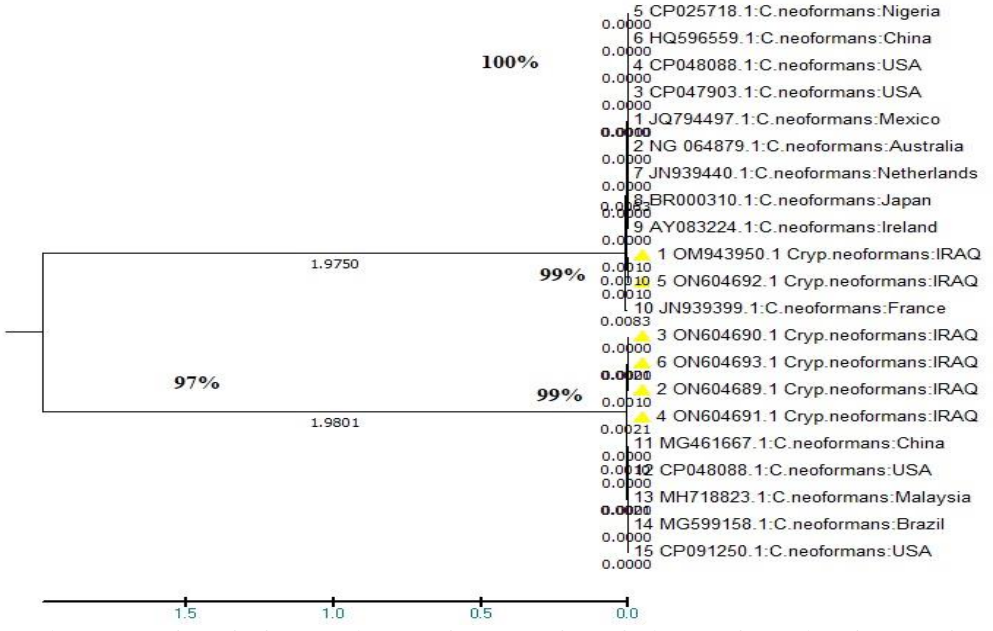

Figure 9: The phylogenetic tree for samples of pigeon dropping have 15

Observation through phylogenetic tree the strains numbers OM943950.1 and ON604692.1 were confirmed by other neighboring sequences since it was found that GenBank isolates under the accession numbers CP048088.1, NG_064879.1 which was deposited from USA and Australia matching is get 99%. While my isolate numbers ON604689.1, ON604690.1, ON604691.1 and ON604693.1 were confirmed by other neighboring sequences since it was found that GenBank isolates under the accession numbers CP048088.1 and MG461667.1 which was deposited from USA and China.

Conclusion

This research confirmed isolation of cryptococcus species other than neoformans from natural habitat of C. neoformans (pigeon droppings) and their related population (pigeon fanciers), so the selective and differential media used in this research recorded isolation of cryptococcus species with 9 (18%) from droppings and 6 (12%) from humans sputum, while PCR assay detect presence at only 9 isolates of C. neoformans divided into 6 (66.6%) in pigeon droppings and 3 (50%) from human sputum, so PCR analysis prove their accuracy and sensitivity and specificity in identification such pathogenic yeast, from the other point of view presence of non neoformans cryptococcus don't deny the importance of cryptococcus species as pathogenic yeast able to cause infection whenever immune deficiency. These results indicate that there may be a risk for human exposure to cryptococcal disease due to inhalation of basidiospores from dropping of pigeon in pigeon houses, especially while cleaning pigeon cages. Therefore, it is risk for the Immune suppression individuals involved in the causation of cryptococcal disease. So that patients with AIDS, diabetes, chronic liver disease, chronic renal disease, prolonged use of steroids and patients who undergo organ transplantation are commonly associated with the development of cryptococcal disease.

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