

Isolation and Identification of *Cryptococcus neoformans* from some Environmental resources in Najaf covernorate

Fatima A. Majbeel

Nabeel S. Majeed

Department of Biology/ College of Science/ University of Kufa

nabeelsahib63@gmail.com

Abstract

The aims of the present study were to isolate and identificate of isolation and identification of *Cryptococcus neoformans* from some environmental resources in Najaf covernor associated with fungal infection. *C.neoformans* represents one of the isolated fungi from environmental resources samples, which may cause many serious problems to human and in immunocompromised patients in particular.

Laboratory isolation and identification of *C. neoformans* a total of (150) environmental resources samples are collected from (6) different areas; (100) samples are obtained from pigeon droppings and (50) samples from soil surrounding the *Eucalyptus* sp. trees and decaying wood were collected, during 2015.

The positive results of cultures (112) (74.66%) isolates of *C. neoformans* were isolated from samples of pigeon droppings and isolates from soil surrounding the Eucalyptus trees and decaying wood samples.

The cultural and biochemical methods were used in *Cryptococcus* spp. Isolation and identification, besides VITEK2 compact system identification kit and CHROM agar candida medium. Also identification by VITEK 2 compact system, showed two speices *C. neoformans* (12) samples and *C. gattii* (3) samples.

The best trust level appeared within potentiality rate of 93%, however the other isolates had a fewer potentiality rate reached 85% and this level was accepted. *Cryptococcus* was identified by conventional tests and yeast which show positive results for the capsule test, urease test, melanin pigment when cultured on methyl dopa culture for a period from 18 – 24 hrs. Then confirmed by VITEK2 compact system identification kit. The results of CHROM agar candida medium test are confirmed the results of the VITEK 2 compact yeast identification system. In this test, the colony color of *Cryptococcus* spp. on the medium are pink for *C. neoformans*.

Key words: *Cryptococcus neoformans*, *Cryptococcus gattii*, isolation and identification, environmental sources, VI TEK2 compact system for identification.

عزل وتشخيص المكورات الخبيثة من بعض المصادر في محافظة النجف

نبيل صاحب مجيد مرزوك

فاطمة عبدالحسين مجبل التميمي

قسم علوم الحياة/ كلية العلوم/ جامعة الكوفة

المستخلص

من خلال دراسة عزل وتشخيص الخمائر المصاحبة لحالات الاصابة بالمكورات الخبيثة، اظهرت نتائج

الدراسة المسحية من نماذج المصادر البيئية المختلفة في بعض مناطق النجف للعام (2015) ابتداءً من شهر

كانون الثاني ، وبواقع 150 عينة من مناطق جغرافية (بيئية) متنوعة، شملت أفضية ونواحي محافظة النجف ، متمثلة بـ (100) عينة من فضلات الحمام، فضلاً عن 50 عينة من التربة المحيطة بأشجار اليوكالبتوس والخشب المتحلل، وبعد العزل المختبري والتشخيص اظهرت خميرة المكورات الخبيثة وينسب عزل في عينات فضلات الحمام تليها نسبة العزل من عينات التربة الملوثة المحيطة بأشجار اليوكالبتوس والخشب المتحلل. استخدمت الطرق الزراعية والكيموحيوية في عزل وتشخيص انواع خميرة المكورات الخبيثة، فضلاً عن الاختبار التوكيدي VITEK2 compact identification و CHROM agar candida medium للتمييز بين انواع خميرة المكورات الخبيثة.

شخصت خميرة المكورات الخبيثة كانت بالاختبارات الروتينية وقد اظهرت العينات نتيجة موجبة لاختبار وجود المحفظة، فحص اليوريا وكانت منتجة لصبغة الميلانين عند زرعها على وسط زهرة الشمس ولمدة 18 - 24 ساعة، كما شخصت الخمائر ايضاً باستخدام وسط الكروم أكار كانديدا حيث ظهرت باللون الوردي الشاحب ثم شخصت العزلات بصورة نهائية بوساطة جهاز الفايترك 2 لغرض التأكد من تشخيص العينات الفطرية المعزولة. اظهر التشخيص بجهاز نظام الفايترك 2، والذي اظهر نوعين من الخميرة *C. neoformans* بعدد 12 عينة و *C.gattii* بعدد 3 عينة و افضل مستوى ثقة ظهر عند نسبة احتمالية 93%، فيما اظهرت بقية العزلات اقل نسبة احتمالية 85% وبمستوى ثقة مقبول. الكلمات الرئيسية: المكورات الخبيثة، العزل والتشخيص، مصادر بيئية، نظام VITEK2 للتشخيص.

Introduction

The term opportunistic fungi is vague. and any fungal species is capable of causing infection under favorable conditions. Over one million fungal species exist in nature. Only a few hundred of them have been associated with human and animal infections. Opportunistic fungi refers to those fungi that normally would cause infections in otherwise healthy people but are able to cause infection under certain circumstances such as immunodeficiency, which includes persons with AIDS, neoplastic disease, extremes of age, immunosuppressive therapy, those undergoing organ transplantation (both hematologic and solid organ) and aggressive surgery. (8). These are fungal infections of the body which occur almost exclusively in debilitated patients whose normal defence mechanisms are impaired.

Cryptococcosis is a disease caused by fungi belonged to the genus *Cryptococcus* spp that infect humans and animals, usually by inhalation of the fungus, which results in lung infection that may spread to the brain , causing meningoencephalitis. The disease was first termed “Busse-Buschle disease” after the two individual who first identified the fungus in 1894-1895.

Classically, cryptococcal infection has been associated with pigeons. Although a few cases have been described with exposure to pigeon excreta prior to development of cryptococcosis, most patients have no history of any direct contact with pigeons. Pigeon breeders have produced antibody titers against *Cryptococcus neoformans*, but no increase rate of infection (7) .

The diagnosis of cryptococcosis is usually performed by a combination of methods. A definitive diagnostic technique is not available yet, in spite of being pursued for more than one century. The methods for the diagnosis of *Cryptococcus* spp comprise tests for isolation and identification, Reliable and sensitive diagnostic tools play a crucial role in the control of Cryptococcosis in environment and human. Currently, diagnosis of *Cryptococcus* spp. is mainly performed by conventional methods such as culture, microscopy and biochemical testing.

The above the objective of this Study was to isolate and identify

- of *C. neoformans*, and its serotypes by the use of traditional the technique from environmental resources comparisons contaminated soil and birds residues.

Materials and Methods

sample collection

In this experiment, 150 different environmental samples were distributed as 100 different sample from the pigeon (*Columba livia* var. *domestica*) droppings, and 50 different samples collected from the soil contaminated with pigeon dropping, collected from several sites in Najaf province during the year 2015. Approximately 10 gm of each sample was placed in separate sterile tubes, and transferred to the Mycology .

The Isolation and identification of the yeast

One gram of the biological material was weighed and placed into tubes each containing (10ml) of sterile saline solution (0.85%Nacl) and (0.05g/l) chloramphenicol , to isolate *Cryptococcus* spp. The specimens were subsequently stirred for three minutes in a vortex apparatus. After stirring, the materials were allowed to stand for five minutes and were then diluted from each suspension with sterile normal saline containing chloramphenicol (1:100 dilutions) . A aliquot (0.1ml) of the supernatant was removed and seeded in triplicate on Sabouraud Dextrose agar. The cultures were incubated at 37°C and anointed dialy for up to 3 days to evaluate the colony morphology. To identify *Cryptococcus* species , the isolates were subjected to morphological and physiological tests, including the production of phenol oxidase on sun flower agar, the detection of urease in ureabase agar media, carbon and nitrogen assimilation. To confirmed the *Cryptococcus* spp., the ViTEK2 compact system were done. Then identification and in order to confirm their diagnosis as they were *C. neoformans*, the following tests had been done, in addition to comparing them with the main characteristics of the reference strain of *C. neoformans* provided by Microbiology Department/College , of Veterinary Medicine, University of Baghdad .

Macroscopic appearance

In this test , the shape, color, consistency, odor, edges of the colony were examined and other apparent characteristics of the yeast colonies that were appeared first of all on the primitive media (Sabouraud dextrose agar).

Microscopic examination

This test was carried out as given below:

- a. One drop of lactophenol cotton blue was put on the slide and then mixed with a yeast colony and then covered with a cover Slide and examined under 40 x lens to determine the cells shape .

b. The second method was done by putting a drop from normal saline or distilled water on the slide, then mixing with a colony from the yeast growth after drying of the slide, a drop from India ink was added, and covered slide with cover, then examined under 40x lens. This test was done for detecting the capsule, which surround the cell of the *cryptococcus*.

Yeasts contain capsule seem to be surrounding by clear hallow as a result of non staining by the stains using in the test, so this test was used to differentiat the yeast producing capsule from those not producing .

- **Gram stain solutions:** This stain was used to determine the response these yeast to Gram stain, since most yeast gives positive results to Gram stain.

The canavanine-glycine-bromothymol blue agar test (Biotyping)

Environmental isolates obtained from different geographical regions, were used. All the isolates were grown on Sabouraud-dextrose medium, (Difco, Detroit, MI, USA), prior to subsequent analysis, the species identification of each sample was determined using the canavanine glycine bromothymol blue agar test (9).

Biochemical examinations

Urea hydrolysis test

Urea agar medium was used to determine the ability of the yeast to consume urea and release the ammonia. The culture was prepare by inoculating a part from the growth by a loop and culturing it following stabbing and streaking method on the slant media then incubated at 37°C for seven days, the changing in color from light orange to the pink or light purple is an indication on the positive result of the test (3).

Melanin production test

Determination was achieved on the yeast ability to produce melanin pigment and phenol oxidase activity. Those was shown through producing small brown colour "pin head" colonies when these yeasts grown on the sunflower seeds agar where the isolates were cultured and incubated at 30 °C for 48 – 72hrs.

Identification by VITEK – 2 Compact systems

(Final) identification for yeast was confirmed by using VITEK – 2 system using AST – YSO6 Cards, This system was used for the identification of microorganisms depending on properties and data analysis about the microorganism. These cards are depending on the principle biochemical testing work, so each card contain primary material measuring different metabolic reactions of the microorganism such as measuring the (acidity, alkalinty, the growth of the microorganism) by suppressing some material, the method included the following step:

1. Preparation of yeast suspension:

The suspension was prepared according to the manufacturer's instructions by transfer suitable amount from the pure yeast colony deals with this study by using a sterile plastic cultural transferor to a sterile plastic test tube containing 3ml physiological saline solution (NaCl 4.50%) mixed gently then the suspension turbidity was test measured by turbidity meter to become near the density of McFarland tube solution no.2.

2. Inoculation of the card:

AST–YSO6 cards were inoculated with prepared colony suspension by suspension and card transfer to the system carrier and putting together in the specific location decided for them, then the card and the suspension were linked with micro – channel, then card number or code number was inserting by the photoscanner after that carrier was placed in a specific chamber air – free vacuum chamber, since vacuum process cause microbial transfer (movement) to the card in addition to distribution them in the wells that content it.

3. Card sealing and incubation:

Conduct channel cutoff mechanically by the system through 10min. the card sealing then transfer to carousel incubator cards incubated in 35.5°C more than 18 hour. Final result for identification recorded after that, which displayed on the computer connected to VITEK2 Compact system.

Molecular typing by REA-RFLP

PCR products of the CAP59 gene was performed followed by restriction enzymes digestion with the two enzymes, BsmFI and HpaII, the digested PCR fragments were separated by run on 1% agarose gel electrophoresis to determine their molecular types.

Results and Discussions

source of isolation different environmental area:

Different environmental samples were collected, which included samples distributed on samples from pigeon droppings and samples from soil and decaying wood in order to determine the presence of *Cryptococcus* spp.

From 150 samples distributed as (100) samples from pigeon droppings and (50) and samples from soil surrounding the *Eucalyptus* sp. trees and decaying wood, in this study showed 112 positive isolates for *C.neoformans* (74.66%)from examination of 6 different environmental sources, as shown in Table (1).

Table (1) shows that the samples isolated from different environmental sources were as follow:

NO	The environmental source	No. of samples isolated from pigeon droppings.	No. samples isolated from soil surrounding the Eucalyptus trees.	Total number Of samples isolated from each location	No. the positive samples	% of positive samples In each location(Na.of all+ve)/(112)	% of positive samples from total number of the isolates(150) in all location s
1	Najaf city	15	3	18	10	8,928	55,555
2	Albuhedarie	25	15	40	25	22,321	62,500
3	Al-Manathra	27	20	47	30	26.785	63,829
4	Al- Hurreia	20	5	25	15	13,392	60.000
5	Al-Mushkhab	25	10	35	15	13,392	42,857
6	Al-Abbaseia	25	10	35	17	15,178	48,571
	Total number	100	50	150	112	100	74,666

One hundred twelve samples (74.66%) out of 150 samples were found to be positive for the presence of *C. neoformans* isolates in 6 investigated location. The highest percentage for positive samples were recorded in Al-Manathera in 30 out of 112

positive samples (26.78%) and Albuhedarie 25 out of 112 positive samples (22.32%). The highest percentage of isolation in an area was in Albuhedarie of 47 samples 25 out of 40 samples (62.50%), Al-Manathera 30 out of 47 samples (63.82%), Al-Abbaseia 17 out of 35 samples (48.57%), Al-Hurreia 15 out 25 samples (60.0%), Al-Mushkab 15 out of 35 samples (42.85%) and Al-Najaf center 15 out of 18 samples (55.55%) as illustrated in (table (1)).

The fungi can be found in a variety of environment, such as plants, air, soil, and water, the contamination associated with lack of hygiene during the handling of birds, and poor equipments used in cleaning, leads to the development of cryptococcosis. In addition to the use of broad-spectrum antimicrobials for a long period, which represented the main factors that precipitates the occurrence of mycotic infection.

C. neoformans can temporarily colonize the intestinal tract of avian species. It can also be found in the guano of asymptomatic birds, either because it was shed from the bird or the droppings provided the nutrients for the yeast organisms to proliferate. This yeast is especially prevalent in droppings from columbic form (eg. Pigeons).

There is an association between *C. neoformans* and pigeon. However, pigeons do not acquire cryptococcosis, most likely because *C. neoformans* cannot grow at the pigeon's normal body temperature of 42°C. According to the recent studies, the environmental habitat of *C. neoformans* appears to be related to trees and plant materials. It is likely that the environmental niche of the fungus is vegetation but that it is easily isolated from avian excreta because it provides good media for growth and can favors the domestic contamination (1). In the current study some of the studied areas showed high isolation rates and this may increase risks of infection among the vulnerable groups due to the domestic contamination in *C. neoformans*. The differences in the isolation rates among the tested locations can be attributed to many factors that can affect the prevalence of the fungus in the excreta of pigeons such as: sunlight exposure to the pigeon habitats due to the susceptibility of this fungus to the ultraviolet radiation, avian alimentary habits and breeders habits such as the way and the frequency of cleaning the coops and the humidity of the samples. In soil, *C. neoformans* may compete with certain microorganisms and can be inhibited by others, such as amoeba, which can devour the yeast. The pigeon excreta offer an advantageous ecological niche for *C. neoformans* in which yeasts can survive when they pass through the pigeon intestinal tract. The yeast cells of *C. neoformans* were found to survive for 2 years in dry pigeon excreta that was protected from the sun. The above mentioned factors are thought to play a role in the ecology and the epidemiology of *C. neoformans* and could explain the failure and the success in isolation of that fungus from the environmental samples.

This study proved the presence of the *C. neoformans* in the pigeon droppings in Najaf with 74.66 % positive isolation in the tested samples. The isolates were able to express the pathogenicity indicators as capsule, pigments production , urease and the ability to grow at 37°C .

Cryptococcus gattii has a more restricted geographical distribution than *C. neoformans* and is predominant in climates ranging from temperate to tropical. In a study on

human clinical cryptococcosis, isolates of *C. gattii* were not found in Austria, Belgium, Denmark, France, Germany, Holland, Italy, Switzerland, and Japan but were identified at an unusually high prevalence in Australia, Brazil, Cambodia, Hawaii, southern California, Mexico, Paraguay, Thailand, Vietnam, Nepal and Central Africa (4). Follow up studies have confirmed the high prevalence of *C. gattii* in tropical and sub-tropical regions including Brazil, Thailand, Papua New Guinea, Venezuela, South Africa and Mexico. Small numbers of human *C. gattii* cases have been reported from India, China, Taiwan, Peru, Argentina, Rwanda, Italy (4). In other reports *C. gattii* has been isolated in Germany from a patient working with wood of imported tropical trees; from environmental samples in Italy, Spain and from Eucalyptus sp. samples in Portugal. It was reported that climatic conditions are related to the distribution of *C. gattii*, it seems therefore that different tolerances of the genotypes/serotypes to environmental conditions, such as climate, may affect their geographic and ecologic distributions (2). Failure for isolation of *C. gattii* from some eucalyptus species in some studies were reported while in others it was proposed that the yeast dispersal is linked to the flowering of the eucalypt trees. In this study could find the *C. gattii* though different potential eucalyptus materials like soil, leaves and flowers were tested. Iraq considered as subtropical country nevertheless Najaf governorate is characterized by a unique situation for Iraq as it is located on the head of the Western Plateau, has the marshes and Al-Euphrates river and all are affecting Najaf climatic conditions, especially in summer that temperature could exceed 47°C, a temperature that is known to cause the death of the *Cryptococcus* species (6).

Identification of *C. neoformans*

The identification of *C. neoformans* was done as primary identification using morphological and biochemical tests, a (Vitek2 compact system).

Morphological and microscopical examination

Initial identification for *C. neoformans* was made by colony characterization often culturing on Sabouraud dextrose agar, *C. neoformans* appeared as creamy colored, smooth, mucoid, convex, and glistening colonies within 2-3 days at 30°C and developed to wrinkled whitish – creamy colonies after further incubation for 7 days as shown in fig.1.



Fig. 1: *C.neoformans* on Sabouraud dextrose agar medium. The mucoid appearance of colonies caused by the polysaccharide capsule..

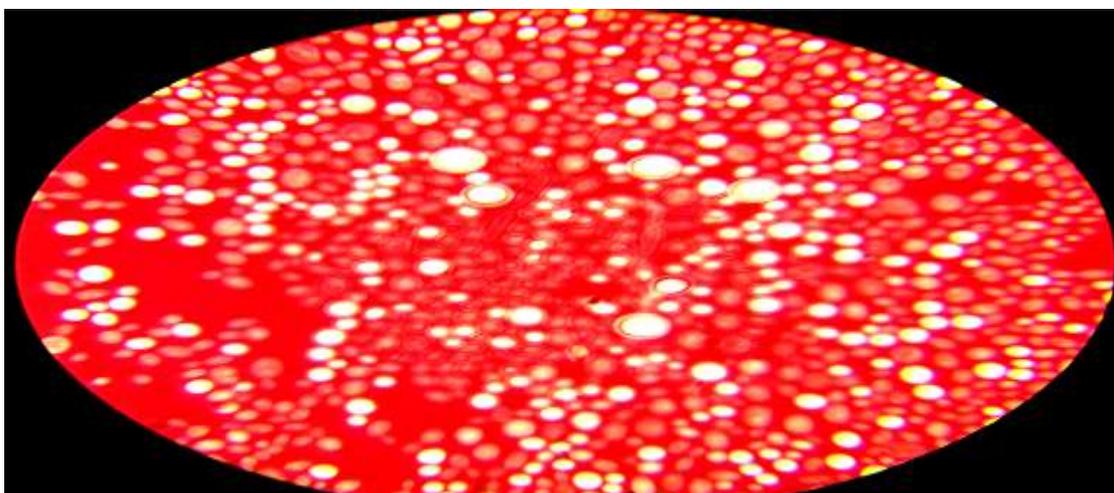


Fig. 2: The microscopical identification of yeasts isolated from different environmental samples by using in diaink (100 X).

C. neoformans produces mucoid colonies on Sabouraud dextrose agar. Microscopically, these organisms were seen as budding yeasts with a narrow neck connecting the mother and daughter yeast cells. The production of a large, mucoid capsule is a unique feature of *Cryptococcus* among fungal organisms. by using the India ink staining, the yeast cells show as oval to spherical in shape cells surrounded by transparency hallow zone from a capsule, and neither with hyphae nor pseudo hyphae

Biochemical tests

Melanin pigment production test:



Fig.3: Melanin production and colony morphology of *C. neoformans* on sunflower seeds agar with 5 days of incubation at 30°-33°C.

The most conformation test for the identification of *C. neoformans* was the use of selective medium sunflower seeds agar, the *C. neoformans* colonies appeared as mucoid appearance with light to dark brown pigmentation which develops with five days of incubation at 30° – 33°C, and this is due to the presence of the enzyme phenoloxidase in *C. neoformans* which is unique among other members of this genus which is responsible of the conversion of phenolic compounds into melanin, the phenoloxidase enzyme is thought to be an important virulence factor as shown in fig. 3.

Urease enzyme production Test:

The *C. neoformans* showed the ability to utilize urea and liberate ammonia by the ability of the yeast to produce urease enzyme and changing the color of the media from yellow to the pink or light purple during 48 hours from incubation at 30° – 33°C as shown in Fig. 4.

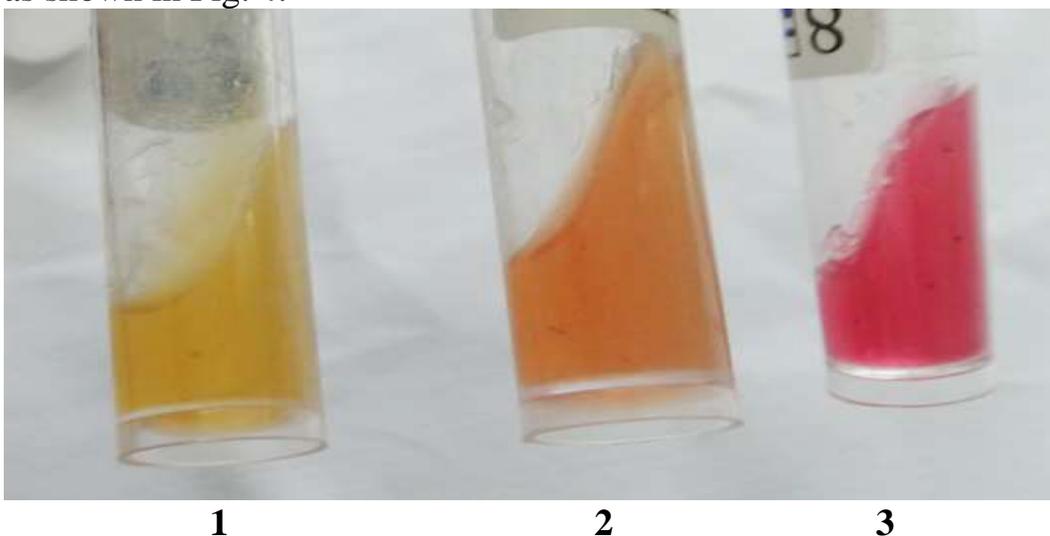


Fig.4: The ability to assimilate urea and liberate ammonia by *C. neoformans* isolates after 2 days of incubation at 30°-33 °C

No.1: Control

No.2: Partial analysis

No.3: complete analysis

Biotyping using canavanine – glycine – bromothymol blue medium (C G B)

The 112 isolates were sensitive to CGB medium and were unable to grow. Thus the isolates were found to be considered as *C. neoformans*.

4-3: Identification of Cryptococcus spp. By VITEK2 COMPACT system:

The results showed that 80 isolates as *C. neoformans* and 25 isolated *C. gattii*; whereas *C. laurentii* was 7, and the best probability ratio was at the *C. neoformans* at 86% at acceptable *identification confidence* level, while the *C. gattii* at 93% probability ratio at very good identification, this were agreement with (5) who explain that the using of VETEK2 compact system was considering useful for *Cryptococcus* identification at the species level .there were relative variation in the biochemical results between the two species, *C. neoformans* and *C. gattii* that the *C. neoformans* showed negative results for the each of the following tests:

L-sorbose assimilation (ISBEa)

Arbutin assimilation (IARAa)

Cellibiose assimilation (dCELa)

Arbutin arylanidas (ARBa)

Tyrosine assimilation (TyrA)

As comparable with the positive result in *C. gattii* for the same tests, whereas positive result were given in L-Rhamnose assimilation test (IRHAa) when it compared with the negative result in *C. gattii* on the other hand *C. gattii* gave positive result for Esculin hydrolysis (ESC) compared with negative result for the same test in *C. neoformans*. More the detailes shown in appendices 3 and 4.

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