

腐生性Cryptococcus neoformansの分離

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Isolation of Saprophytic *Cryptococcus neoformans*

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ABSTRACT. Isolation of *Cryptococcus neoformans* was carried out on sunflower seed agar medium (SFA) and Sabouraud dextrose agar (SDA). Out of 346 environmental substrates (133 fruits, 107 avian excreta, 91 vegetables and 15 wooden scrapings) tested, 3 specimens were positive for *C. neoformans*. The positive isolations came from the fruits of 2 banana (*Musa sapientum*) and a potato tuber (*Solanum tuberosum*). The pathogen could not be demonstrated in 107 samples of avian droppings and 15 of wooden materials. All the 3 isolates of the yeast were obtained on SFA, while they were not cultured on the plates of SDA with chloramphenicol which were badly contaminated with rapidly growing molds, yeasts and bacteria. To the present author's knowledge, this appears to be the first reports of the isolation of this pathogenic basidiomycetous yeast from contaminated fruits of banana. We suggest more comprehensive ecological surveys to search for environmental niche of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* as the latter variety is also implicated in the etiology of cryptococcosis.—**KEY WORDS:** *Cryptococcus neoformans*, environmental substrate, saprophytic reservoir, sunflower seed agar.

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Cryptococcus neoformans, a well known animal and human opportunistic pathogen, grows in nature as saprophyte and has been recovered from a wide variety of environmental materials [2, 6, 7].

In recent years, *C. neoformans* var. *gattii* has been reported as a cause of human cryptococcosis [5, 8, 9, 10]. Hitherto, the variety *gattii* has not been recovered from the saprophytic sources [11].

This initiated the present study to investigate the environmental distribution and variety of *C. neoformans*.

MATERIALS AND METHODS

Sample collection: During May, 1989 to February, 1990, a total 346 diverse types of environmental materials comprising 133 fruits (83 bananas, 19 apples, 17 oranges and 14 pears), 107 avian excreta, 91 vegetables (51 potatoes, 13 carrots, 10 pimentoes, 8 tomatoes, 6 cucumbers and 3 cabbages)

and 15 wooden scrapings, collected randomly from various localities in Tokyo, was screened mycologically for the presence of *C. neoformans*.

Isolation technique: a) Avian excreta: One gram of test material was suspended in 9 ml of sterile saline in a sterilized glass test tube and mixed thoroughly. From this suspension, plates of sunflower (*Helianthus annuus*) seed agar medium (SFA) [12] with chloramphenicol (0.05 mg/ml) and Sabouraud dextrose agar (SDA) with chloramphenicol (0.05 mg/ml) streaked directly with a sterilized cotton swab.

b) Wooden scrapings: The pour plate method was employed to recover the yeast from wooden scrapings obtained from the nest boxes and perches of 2 aviaries at a local zoological park. Disposable sterile polystyrene plate (90×15 mm) containing 1 ml supernate of wooden dust was overlaid with 20 ml of melted SFA and SDA.

c) Fruits and vegetables : A sterilized

cotton swab moistened in sunflower seed broth was rubbed on the outer surface of the freshly purchased fruits and vegetables, and then the swab was streaked directly onto the plates of SFA and SDA.

Identification: The inoculated Petri dishes of SFA and SDA were incubated at 25°C and examined daily for 3 weeks before describing them as negative for *C. neoformans*. Each isolate of yeast-like organisms on SFA and SDA was subcultured, purified and subjected to detailed morphological and biochemical tests, as well as the variety determination [4] and sexual compatibility [9].

Effect of avian intestinal bacteria on in vitro growth of C. neoformans: For this qualitative assay, 3 strains of *C. neoformans* (animal, human and natural) were grown on SDA for 2-day at 37°C. A heavy loopful of 48 hrs old growth of yeast was suspended in 4 tubes containing 5 ml of brain heart infusion (BHI) broth. The same quantity of 2 days old intestinal bacteria recovered from the fresh avian excreta, was inoculated in 3 tubes of BHI broth already seeded with *C. neoformans*. The other tube number 4 served as control which did not contain bacteria. This suspension was mixed thoroughly and incubated at 37°C for 7 days. After 1 week of incubation, 0.1 ml of the suspension from all 4 tubes was streaked onto the plates of SFA, and the inhibition was defined as no growth on SFA after 1 week of inoculation at 25°C.

In vitro colonization of fruits and vegetables with C. neoformans: Six different varieties of vegetable matters, namely banana, cabbage, carrot, cucumber, pimento and potato purchased from the local shops in Tokyo were used for this *in vitro* experiment. Each test sample was thoroughly washed in running tap water and sliced with scalpel. One set of the substrate was autoclaved in glass Petri dishes for 10 minutes and the other set was left unsterilized in the plates.

A known strain of *C. neoformans* recovered from the sputum of an immunocompromised patient was cultured on SDA for 48 hrs at 37°C. The growth was suspended in 1 ml of sterile saline to contain 1×10^6 cells. From this mixture, 0.1 ml was inoculated *in vitro* onto the autoclaved and unautoclaved test materials. The experimentally seeded plant substrates were incubated at 25°C for 1 week. The inoculated plates were daily inspected for the *in vitro* growth of *C. neoformans*.

RESULTS

Of 346 environmental samples investigated mycologically, 3 (0.86%) yielded *C. neoformans* on SFA, suggesting a very low prevalence of the yeast (Table 1). The positive isolations came from the contaminated fruits of 2 banana (*Musa sapientum*) among 83 samples and a potato tuber (*Solanum tuberosum*) among 51 samples.

Table 1. Prevalence of *C. neoformans* var. *neoformans* in different environmental substrates

Substrates	Number of samples tested	Number of samples yielding the yeast of SFA
Fruits	133	2
Avian excreta	107	0
Vegetables	91	1
Wooden scrapings	15	0
Total	346	3

The remaining 343 specimens were consistently negative for *C. neoformans*. All the 3 isolates of this basidiomycetous yeast were cultured on SFA (Table 1). However, SDA plates inoculated with the same specimens produced no isolates of *C. neoformans* because of the contamination with rapidly growing molds, yeasts and bacteria. The number of colonies of the yeast which grew on SFA varied from 1 to 3, indicating that the plant substrates were in a very low contamination of the organism.

All *C. neoformans* isolates were identified variety *neoformans* as determined by D-proline assimilation technique. Sexual compatibility studies indicated that they belonged to mating type "α" of *Filobasidiella neoformans*.

C. neoformans failed to reisolate in 1 week of incubation in BHI broth when inoculated with avian intestinal organisms. However, *C. neoformans* grew luxuriantly in the control tube which contained only known quantity of *C. neoformans* cells. Out of 6 tubes of vegetable substrates tested, only 5 varieties namely banana, cabbage, carrot, cucumber and potato showed good luxuriant isolation of *C. neoformans* when experimentally seeded under sterile condition. No growth was observed on the autoclaved pimento. On the contrary, unsterilized plant materials failed to support the growth of the yeast because of their easy contamination by the rapid growth of saprophytic organisms.

DISCUSSION

In the past many ecological and epidemiological studies have been shown that *C. neoformans* is widely distinguished in the environment and pigeon excreta serves as the chief reservoir of this pathogenic yeast [1, 11, 15]. In the present investigation, *C. neoformans* could not be recovered from any of the 107 samples of

fresh avian droppings. On the contrary, the vegetable matters (2 banana and 1 potato) yielded very low contamination (1–3 colonies) of *C. neoformans* var. *neoformans* "α" mating type SFA at 25°C. The results of this study suggest that the recovery of *C. neoformans* from the fresh plant substrates is extremely difficult without the use of a highly sensitive selective medium. The earlier investigators reported only sporadic recovery of *C. neoformans* from plant sources [3, 13, 14].

The consistent failure to isolate *C. neoformans* from the fresh avian excreta may possibly be due to the inhibition of this yeast by other intestinal microflora of the birds droppings as amply demonstrated *in vitro* by the inability to reisolate the pathogen from the mixed bacterial culture inoculations along with *C. neoformans*. These findings lend support to the possibility that the persistence of *C. neoformans* in avian excreta is adversely influenced by other microorganisms. Similar observations have been recorded by previous workers that normal avian bacterial flora namely *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* resulted into complete inhibition of the *in vitro* growth of *C. neoformans* [1, 13].

The results of *in vitro* colonization of vegetable matter by *C. neoformans* showed that plant substrate can support the growth of the yeast under defined laboratory conditions. However, experimental seeding of the same vegetables and fruits with the pathogen under unsterilized condition failed to exhibit the growth of *C. neoformans* as contaminated by a number of saprophytic organisms. The easy contamination of the vegetable matter by the rapidly growing molds and bacteria mask the growth of this basidiomycetous yeast and therefore render its isolation extremely difficult [13]. It is suggested that ecological studies on the natural occurrence of *C. neoformans* in various environmental niches should be

carried out on SFA which is a very sensitive and highly specific selective medium for *C. neoformans*.

The perusal of available literatures indicate that this is the first report of the isolation of *C. neoformans* var. *neoformans* from the fruits of banana (*Musa sapientum*).

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要 約

腐生性 *Cryptococcus neoformans* の分離：Mahendra Pal・恩田千景・長谷川篤彦（東京大学農学部獣医内科学教室）——各種検体346例（果実 133, 鳥の排泄物 107, 野菜 91, 木の削りかす 15）中バナナ2例, ジャガイモ1例から *Cryptococcus neoformans* var. *neoformans* を分離した。クロラムフェニコール添加サブロー・デキストロース培地では急速に発育する雑菌の汚染が著しく分離不可能であったが, ヒマワリ培地では褐色を呈するコロニーを採取することにより分離することができた。バナナからの分離報告は初めてであり, 本病原体については *C. neoformans* var. *gattii* もふくめて野外分布を知る上で, より広範囲な疫学的調査が必要と考える。