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First report of isolation of *Cryptococcus neoformans* var. *neoformans* from avian excreta in Kathmandu, Nepal

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Summary

This paper delineates the first report on the saprophytic distribution of *Cryptococcus neoformans* var. *neoformans* in the city of Kathmandu, Nepal. Twenty-eight samples of old and dry pigeon droppings collected from different sites in Kathmandu were investigated for the presence of *C. neoformans* by employing a dilution technique. The organism was isolated from seven (25%) of the specimens, representing four of the ten collection sites. All of the isolates were recovered on Pal's medium (sunflower seed agar) by observing light to dark brown coloured colonies of *C. neoformans*. However, no isolation could be acheived on Sabouraud medium as all the plates were badly contaminated with rapidly growing moulds. The microscopic morphology of the cultures in PHOL stain revealed circular to val, single or budding yeast cells with thin capsules. Detailed typing of all environmental strains indicated that they belonged to the variety *neoformans* and α mating type of *Filobasidiella neoformans*. The results of this study demonstrated that Pal's medium is an excellent differential medium for the screening of environmental specimens and *C. neoformans* var *neoformans* is prevalent in the environment of Kathmandu.

Key words *Cryptococcus neoformans*, Environment, Nepal, Pal's medium

Primera comunicación del aislamiento de *Cryptococcus neoformans* var. *neoformans* de excretas de ave en la ciudad de Kathmandu, Nepal

Resumen

Se presenta la primera comunicación del aislamiento de *Cryptococcus neoformans* var. *neoformans* en la ciudad de Kathmandu, Nepal, que demuestra la distribución de la forma saprofítica en este área del mundo.
Veintiocho muestras de excrementos viejos y secos de palomas fueron recogidas de diferentes partes de Kathmandu, investigándose la presencia de *C. neoformans* por cultivo mediante la técnica de dilución. La levadura fue aislada de siete de las muestras (25%) en cuatro de los lugares examinados. Todos los aislamientos se efectuaron en el medio de Pal (semillas de girasol) por observación de colonias de color marrón. No fue posible el aislamiento con el medio de agar de Sabouraud, ya que todas las placas resultaron contaminadas por el rápido crecimiento de mohos.
La morfología microscópica de los cultivos con la tinción de PHOL reveló levaduras circulares u ovaladas con gemación, rodeadas de una fina cápsula. La tipificación de todas las cepas, basado en la no asimilación de D-prolina, demostró que todas pertenecían a la variedad *neoformans*. El ensayo de reproducción sexual fue positivo con el tipo α de *Filobasidiella neoformans*. Los resultados de este estudio demuestran que el medio diferencial de semillas de girasol es excelente para el estudio de materiales naturales y que *C. neoformans* var. *neoformans* es prevalente en Nepal.

Palabras clave *Cryptococcus neoformans* var. *neoformans*, Ambiente, Nepal, Medio de Pal

Cryptococcus neoformans is the principal etiological agent of cryptococcosis that has a world wide distribution [1-3]. The pathogen occurs as a saprobe in a wide variety of natural substrates including avian excreta, soil and wood [4-9]. The yeast has been frequently recovered from pigeon droppings in many countries of the world but not Nepal [1]. Therefore, the present study was conducted on the environmental distribution, variety and mating types of *C. neoformans* in Nepal

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MATERIALS AND METHODS

Twenty-eight samples of dried and old pigeon droppings were collected with wooden spatulas and placed in clean polythene bags from ten different locations in Kathmandu, the capital of Nepal. They were processed in the laboratory of Veterinary Public Health, Anand, India. One gram of specimen from each site was suspended in sterilized glass bottle containing 99 ml of sterile physiological saline (0.85% NaCl) supplemented with chloramphenicol (10 mg/ml). The mixture was left at room temperature for about 10-15 min, then shaken manually for 4-5 min and later incubated at 37°C for one hour. Ten-fold serial dilutions were made and 0.1 of each dilution was streaked onto duplicate plates of Sabouraud dextrose agar with chloramphenicol (0.05 mg/ml) and Pal's medium (pulverized sunflower seed 45 g, agar 20 g, chloramphenicol 500 mg, distilled water 1000 ml). The former medium was kept at 37°C while the later was incubated at 25°C. Each inoculated plate was examined daily for yeast growth and the number of colonies of strains growing at 37°C with capsule, urease positive and lactose positive identified as *C. neoformans* were counted after 3-4 days. The average number of colony forming units per gram of specimen indicated the presence of *C. neoformans*. The D-proline assimilation test was used to determine the varietal status of the environmental isolates [10]. *In vitro* sexual crossings were conducted on modified Pal's medium that contained 22.5 g pulverized sunflower seed, 1.0 g KH_2P0_4, 0.5 g $MgS0_4$, 20 g agar and 1000 ml distilled water. Microscopic morphology of the isolates as well as growth on Pal's medium were studied in PHOL's stain (3 ml glycerol, 0.3 ml, 3% aqueous solution of methylene blue and 5 ml of 4% aqueous solution of formaldehyde) [11]

RESULTS

Out of the 28 samples of the pigeon droppings, *C. neoformans* was recovered from seven specimens giving a prevalence of 25%. The yeast was isolated from four of the ten sites examined in Kathmandu. The average number of *C. neoformans* cells isolated from the pigeon excreta varied from $1,5 \times 10^4$ to $2,0 \times 10^5$ cfu/g of specimen. All the isolations were made only on Pal's medium at 25°C by observing light to dark brown pigmented colonies of *C. neoformans*. In contrast, the pathogen could not be isolated on Sabouraud medium at 37°C as all of the inoculated plates were badly contaminated with fast growing moulds that masked the growth of *C. neoformans*.

All the seven isolates grew well on Sabouraud medium at 37°C, hydrolyzed urea, but failed to utilize potassium nitrate ($KN0_3$) and lactose. The yeast cells in PHOL stain under light microscopy showed many spherical to few oval thinely encapsulated yeast cells, with and without budding. None of the isolates assimilated D-proline and therefore, were identified as variety *neoformans*.

In vitro sexual crossings gave rise to white, cottony luxurient growth around the margins of the paired colonies when mated with α standard tester strain of *Filobasidiella neoformans* var. *neoformans* on Pal's medium at 20°C for 7-15 days.

The microscopic morphology of the growth from the mated colonies in PHOL stain revealed basidia, basidiospores and dikaryotic hyphae with clamp connections. No sexual compatibility was observed with *F. neoformans* var. *bacillispora.* The results indicated that all the environmental strains of *C. neoformans* were of the α mating type of *F. neoformans* var. *neoformans*.

DISCUSSION

The ecological relationship of *C. neoformans* was first reported by Emmons [12] from USA by isolating this yeast fron the droppings of the pigeon *Columbia livia*. Subsequently many researchers from different regions of the world confirmed this finding [1,3,6,8,13,14]. The recovery of *C. neoformans* from the pigeon excreta for the first time in Nepal established that avian habitats serve as an important saprobic reservoir for this opportunistic pathogen. However, further studies on the occurrence of *C. neoformans* in a wide variety of environmental materials should be undertaken to reveal the ecological niches of this basidiomycetous yeast in Nepal.

The findings of this investigation indicated that the isolation of *C. neoformans* from environmental sources is extremely difficult on conventional media like Sabouraud agar. Therefore, a differential medium should be employed for investigating the prevalence of *C. neoformans* in saprophytic materials. We were successful in recovering *C. neoformans* in 25% of the samples of pigeon dropping on Pal's medium. The development of brown coloured colonies on Pal's medium within 3-4 days at 25°C resulted in the rapid isolation and quick presumptive identification of the yeast. As Pal's medium is highly specific, very sensitive, less expensive and easily available, its routine use as an excellent selective medium will help microbiologists in ecological and epidemiological studies of *C. neoformans* that has emerged as an important opportunistic pathogen producing life threatening disease in immunocompetent and immunocompromised hosts particularly AIDS patients [2,14].

All of our environmental strains were found to be of the variety *neoformans*. We did not detect variety *gattii* in the droppings of the pigeon (*Columbia livia*). Our observation is consistent with the findings of earlier investigators who reported the predominance of variety *neoformans* in avian excreta [1,14,15-17]. Since variety *gattii* serotype B has been recorded earlier from human clinical specimens in Nepal [18] it would be advisable if a thorough search of indigenous *Eucalyptus* spp. trees is made by employing Pal's selective medium. However, variety *gattii* is considered to be associated with *Eucalyptus camaldulensis* [19] and *Eucalyptus tereticornis* [20,21].

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