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Isolation of *Cryptococcus neoformans* var. *gattii* from *Eucalyptus camaldulensis* in India

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***Cryptococcus neoformans* var. *gattii* has an ecological association with five *Eucalyptus* species: *E. blakelyi*, *E. camaldulensis*, *E. gomphocephala*, *E. rudis*, and *E. tereticornis*. After human infections due to *C. neoformans* var. *gattii* were diagnosed in the states of Punjab, Himachal Pradesh, and Karnataka, India, a study was undertaken to investigate the association of *C. neoformans* var. *gattii* with Indian eucalypts, especially in the state of Punjab. A total of 696 specimens collected from *E. camaldulensis*, *E. citriodora* and *E. tereticornis* (hybrid) trees were examined for the presence of *C. neoformans* var. *gattii*. Flowers from two trees of *E. camaldulensis* in the Chak Sarkar forest and one from the village of Periana near the Ferozepur area yielded five isolates of *C. neoformans* var. *gattii*. The origin of the trees could be traced to Australia, thus providing evidence that the distribution of *E. camaldulensis* correlated with the distribution of human cryptococcosis cases caused by *C. neoformans* var. *gattii* in northern India.**

Meticulous work by Ellis and Pfeiffer (1–3, 9, 10) has shown that *Cryptococcus neoformans* var. *gattii* (serotypes B and C) is a biotrophic, basidiomycetous yeast associated with *Eucalyptus camaldulensis* (river red gum) and *E. tereticornis* (forest red gum), two closely related species of eucalypts. Exposure to trees that harbor *C. neoformans* var. *gattii* is necessary for infections to occur in humans and animals (3). Recently, Pfeiffer and Ellis (8) showed that three additional eucalypts, namely, *E. blakelyi* (Blakely's red gum), *E. gomphocephala* (tuart), and *E. rudis* (flooded gum), also serve as abodes for *C. neoformans* var. *gattii. C. neoformans* var. *gattii* appears to have been exported from Australia to other countries on contaminated seeds of *E. camaldulensis* and other eucalypts bearing propagules of the fungus. The first isolation of *C. neoformans* var. *gattii* from *E. camaldulensis* trees outside Australia was made in 1991 by Pfeiffer and Ellis (9) from eucalypts growing near Fort Point, San Francisco, Calif. More recently, Montagna et al. (6) reported the first isolation of *C. neoformans* var. *gattii* (serotype B) from *E. camaldulensis* in Italy.

After we first reported the occurrence of *C. neoformans* var. *gattii* causing chronic cryptococcal meningitis in three patients from northern India (7), we studied four additional human cases of cryptococcosis caused by this variety. The geographical distribution of these cases revealed that the patients came from the northern states of Punjab and Himachal Pradesh and from the state of Karnataka in southwestern India. A study was therefore undertaken to investigate the possible association of *C. neoformans* var. *gattii* with the eucalypts growing in Punjab. Australian eucalypts were first introduced into Punjab in 1860. The most commonly grown species of eucalypts in that state are *E. citriodora*, *E. globulus*, and *E. tereticornis. E. camaldulensis* was introduced by the Punjab Forest Department of Research and Training, Hoshiarpur, at the Chak Sarkar forest on an experimental basis in 1982. The seeds were imported from Australia. The saplings were then planted in the Phillaur Reserve forest, Periana village, Patiala Beed Moti Bagh, and two farms at Hoshiarpur in 1983. This report describes the isolation of *C. neoformans* var. *gattii* from *E. camaldulensis* trees in Punjab.

A total of 696 samples of air under eucalypt trees (236), flowers (118), fruits (35), leaves (50), bark (61), debris collected under the trees (90), soil underneath trees (98), and the combs of wild bees' nests (8) were collected over a period of 2 years (1995 and 1996) from three species of *Eucalyptus* trees, namely, *E. tereticornis* (hybrid) and *E. citriodora* from Chandigarh and surrounding areas (11, 12) and *E. camaldulensis* from Patiala, Hoshiarpur, Phillaur, and Ferozepur, all in Punjab (Table 1; Fig. 1).

Air sampling was done by using a slit air sampler (Micro Med, Dynamicro, Bombay, India). The sampler contained strips impregnated with niger seed agar medium, which had been prepared in the laboratory (4). The sampler had an air volume intake of 40 liters/min and was operated for 5 min at each site. The air sampling was carried out below the canopy of plantations of *Eucalyptus* trees. The niger seed agar strips were removed from the sampler after each exposure and were incubated at 30°C for 15 days. They were observed daily for developing brown, yeastlike colonies. All suspected colonies were subcultured on Sabouraud glucose agar. Other eucalypt samples (Table 1) were collected in zip-lock plastic bags and transported to the laboratory. On the following day, 5 to 10 g of each sample was removed and mixed with 25 ml of sterile distilled water in 250-ml Erlenmeyer flasks. The flasks were vigorously shaken, and their contents were allowed to settle for 30 min. Subsequently, a 0.2-ml aliquot of the supernatant from each flask was aseptically pipetted onto plates containing niger seed agar. The cultures were incubated in the dark at 30°C for 15 days and were observed daily. All suspected brown, moist colonies were subcultured on Sabouraud glucose agar. All isolates were first tested for urease production and growth at 37°C. The positive isolates were then identified by carbohydrate assimilation and fermentation tests (4) and by API 20C (bioMerieux, Hazelwood, Mo.) testing. The isolates identified

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TABLE 1. Distribution of *Eucalyptus* samples in Punjab tested for *C. neoformans* var. *gattii*[a]

Location	Tree species	No. of samples tested (no. positive) from:							
		Air (n = 236)	Flowers (n = 118)	Fruits (n = 35)	Leaves (n = 50)	Bark (n = 61)	Debris (n = 90)	Soil (n = 98)	Bee nests (n = 8)
Chandigarh	*E. tereticornis*	75	50	15	35	25	40	53	5
	E. citriodora	5	3	5	0	0	5	9	0
Patiala	*E. camaldulensis*	20	3	2	10	7	7	10	0
Hoshiarpur									
Dadiala	*E. camaldulensis*	15	5	3	0	5	7	5	0
Tootan Do Sarkan	*E. camaldulensis*	35	15	7	5	3	6	5	0
Pillaur	*E. camaldulensis*	26	11	2	0	6	6	6	2
Ferozepur									
Periana	*E. camaldulensis*	25	11 (1)	0	0	3	5	3	1
Chak Sarkar Forest	*E. camaldulensis*	35	20 (4)	1	0	12	14	7	0

[a] See the text for complete descriptions of samples and locations.

as *C. neoformans* were further tested on canavanine-glycine-bromthymol blue (CGB) agar (5) for the differentiation of *C. neoformans* var. *gattii* (serotypes B and C) and *Cryptococcus neoformans* var. *neoformans* (serotypes A and D). Five isolates of *C. neoformans* var. *gattii* that were positive on CGB agar were then sent to Wiley A. Schell at the Medical Mycology Research Center, Division of Infectious Diseases and International Health, Duke University Medical Center, Durham, N.C., for determination of their serotypes.

All isolates identified as *C. neoformans* var. *gattii* were further tested for pathogenicity. A suspension of each isolate (10^5 to 10^6) in saline was injected into the tail veins of two outbred Swiss albino mice. All the mice died 10 to 15 days after injection. The mice were autopsied, and impression smears of the brains were examined for the presence of encapsulated yeast cells.

Of the 696 samples tested, only flower specimens from three *E. camaldulensis* trees, collected near the Ferozepur area,

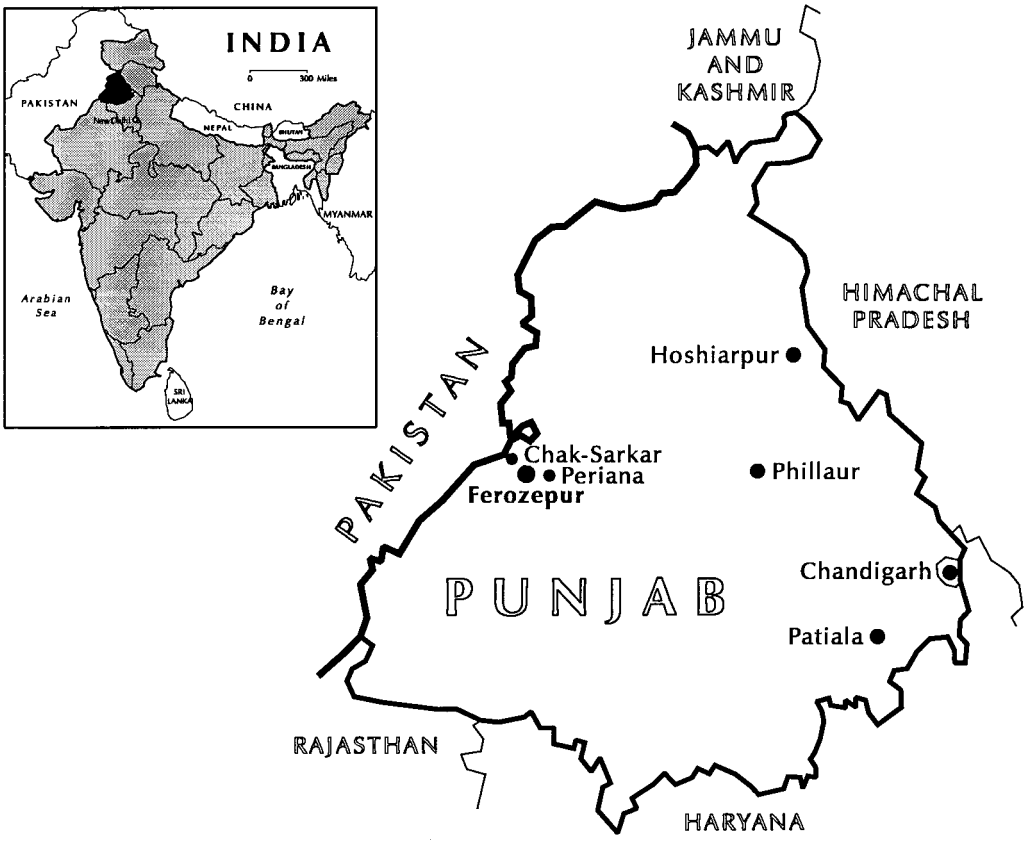

FIG. 1. Map of Punjab, India, showing location of *E. camaldulensis* trees positive for *C. neoformans* var. *gattii.*

yielded *C. neoformans* var. *gattii*. Four isolates (Centers for Disease Control and Prevention strains B-5763, B-5765, B-5787, and B-5788) were recovered from the flowers of two *E. camaldulensis* trees from the Chak Sarkar forest, and one isolate (B-5764) was recovered from flowers of an *E. camaldulensis* tree in the village of Periana (Fig. 1). We were able to determine that the seeds of the two trees growing in the Chak Sarkar forest originally came from Australia, one from the Pente Cort River area and the other from Irvine Bank. The village of Periana is located about 20 km northeast of Ferozepur (Fig. 1). Seeds from the *E. camaldulensis* trees from the Chak Sarkar forest had been planted on a farm in Periana. The tree that yielded *C. neoformans* var. *gattii* was a tree on the same farm. By analysis with the Iatron Crypto 25 test kit, the five isolates were found to be serotype B. All five isolates were pathogenic for mice, and brain smears from infected mice showed numerous encapsulated yeast cells.

The successful isolation of *C. neoformans* var. *gattii* from *E. camaldulensis* in Punjab represents the first Indian isolation of this variety from *E. camaldulensis* imported from Australia and provides support for Ellis and Pfeiffer's observations (2, 3) that this basidiomycete has been exported from Australia on contaminated seeds or seedlings of *E. camaldulensis*. Further studies are planned to investigate the geographic distribution of this yeast in India and its possible association with the *E. tereticornis* hybrid that has become one of the most common eucalypt species in the low-rainfall areas of India (11, 12).

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