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NOTES

Natural Environmental Sources of *Cryptococcus neoformans* var. *gattii*

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We sought evidence for new environmental sources of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA (RAPD) analysis of isolates from 29 animals with a restricted territorial range in five Australian states. Twenty-three of the 29 isolates and 45 of 45 eucalypt isolates tested previously exhibited one RAPD profile, VGI. RAPD profile VGII was identified in 6 of 17 isolates from domesticated species but in none of 12 native species. Four VGII isolates originated from an area of Western Australia with no natural stands of known eucalypt host, indicating the existence of at least one unrecognized natural source of *C. neoformans* var. *gattii*.

Cryptococcus neoformans var. *gattii* has a predilection for the respiratory and nervous systems of apparently healthy humans (13); domestic animal species, such as cats, dogs, and horses (11, 12, 15); and Australian marsupial possums (10) and koalas (17). Human disease is endemic in Australia (4), Papua New Guinea (9), and Southern California and parts of Africa, India, Southeast Asia, Mexico, Brazil, and Paraguay (7).

The eucalypt species *Eucalyptus camaldulensis* and *Eucalyptus tereticornis* constitute the only known environmental niche of *C. neoformans* var. *gattii* (14). Evidence for an epidemiological association between this cryptococcal habitat and human infection is circumstantial. There is a correlation between the global distribution of human infection with *C. neoformans* var. *gattii* and the two species of eucalypt, and environmental searches conducted in Australia and elsewhere have identified no other natural source. Analysis of Australian isolates of clinical and eucalypt origin by random amplification of polymorphic DNA (RAPD) has revealed that all eucalypt isolates ($n = 45$) and 92% of clinical isolates ($n = 48$) exhibit a single major RAPD profile, designated VGI (16). These data strengthen the epidemiological link between eucalypt-derived isolates and human infection in Australia, but important questions remain. Neither *E. camaldulensis* nor *E. tereticornis* occurs naturally in southwest Western Australia (WA) (2), an area in which human cryptococcosis due to *C. neoformans* var. *gattii* has been reported. In the Northern Territory, a variety of *E. camaldulensis*, *E. camaldulensis* var. *obtusa*, is found, but its habitat does not include all of Arnhem Land (2), the site of several cases of human cryptococcosis due to *C. neoformans* var. *gattii* in Australian Aboriginal people reported recently (6), and initial environmental searches in the area have been negative (3). The location in which infection is acquired is rarely identifiable in humans because of their propensity to travel widely and the relatively long incubation period of the disease. In contrast, animals generally reside within a restricted territorial range. To seek evidence for additional environmental sources of *C. neoformans* var. *gattii*, we applied the RAPD technique to cryptococcal isolates from 29 animals in five Australian states. Three Australian and three American eucalypt isolates were included for comparison.

Cryptococcal isolates were stored in serum broth at −70°C and subcultured on Sabouraud dextrose agar. The isolates were identified and biotyped by standard techniques (8). Serotyping was performed by using the Crypto Check agglutination test (Iatron Laboratories Inc., Tokyo, Japan). DNA extraction, primers, and the RAPD method have been described in detail previously (1, 16). Major RAPD profiles (VGI, VGII, etc.) were distinguished from each other by a reproducible difference in band patterns generated by each of the seven primer pairs tested. Subtypes within a major profile yielded identical band patterns with four to six of the primer pairs and a reproducible difference in one or more bands constituting the RAPD pattern with one to three primer pairs (16).

Individual animal data are shown in Table 1, and the geographical distribution of animals is shown in Fig. 1. Except for the two horses, none of the animals had resided outside a restricted range. The horses (one yielded a cryptococcal VGI profile, and one yielded a VGII profile) were thoroughbred geldings that resided during their racing careers in areas of WA containing *E. camaldulensis* or *E. tereticornis*. The 11 koalas were resident in sanctuaries or zoos within their natural range.

Serotyping indicated that all isolates were type B. Representative examples of the three major RAPD profiles, VGI, VGII, and VGIII, and of the animal isolates are shown in Fig. 2 and in Fig. 2 and 3, respectively. Among the animal isolates, RAPD profile VGI predominated (23 of 29 [79%]), with the remaining 6 isolates being assigned to profile VGII.

As indicated in Table 1, 12 of 12 (100%) isolates from indigenous native species were assigned to profile VGI,

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TABLE 1. Animal isolates of *C. neoformans* var. *gattii*

Case no.	Animal species	Location[a]	RAPD profile	Site of disease
1–3	Koala	NSW	VGI	Colonized[b]
4–6	Koala	QLD	VGI	Colonized[b]
7	Koala	SA	VGI	Colonized[b]
8	Koala	QLD	VGI	Ovary
9	Koala	NSW	VGI	Meninges
10	Koala	NSW	VGI	Skin, lymph nodes
11	Koala	NSW	VGI	Nasopharynx
12	Echidna	TAS	VGI	Lung
13	Cat	NSW	VGII	Nasal cavity
14	Cat	NSW	VGI	Nasal cavity, skin
15	Cat	NSW	VGI	Nasal cavity
16	Cat	NSW	VGII	Nasal cavity
17	Cat	NSW	VGI	Nasal cavity
18	Cat	NSW	VGI	Nasal plane, nasal cavity
19	Cat	NSW	VGI	Pyothorax
20	Dog	QLD	VGI	Nasal cavity
21	Dog	NSW	VGI	Nasal cavity, frontal sinus
22	Dog	NSW	VGI	Nasal cavity, periorbital region
23	Dog	NSW	VGI	Nasal plane, lung
24	Sheep	WA	VGII	Nasal cavity, brain, meninges
25	Sheep	WA	VGII	Nasal cavity, brain, meninges
26	Sheep	WA	VGII	Nasal cavity, brain, meninges
27	Horse	WA	VGI	Lung
28	Horse	WA	VGII	Lung
29	African grey parrot	SA	VGI	Nasal cavity

[a] SA, South Australia; TAS, Tasmania.
[b] *C. neoformans* var. *gattii* was isolated from superficial body sites or under claws.

whereas of 17 isolates from nonnative species, 6 (35%) were assigned to profile VGII ($P = 0.03$, Fisher's exact test). There was a preponderance of isolates of profile VGII in WA (4 of 5 compared with 2 of 16 in New South Wales [NSW] and 0 of 8 in the other three states), which persisted when the 12 isolates from Australian native animals (none of which were from WA) were excluded from the analysis (4 of 5 versus 2 of 12; $P = 0.03$, Fisher's exact test). A single environmental isolate of *C. neoformans* var. *gattii* was obtained from WA, from plant debris along the fence line of a paddock containing sheep infected with the same biotype. The RAPD profile of this isolate was VGII, identical to that of the sheep isolates.

The focus of infection with *C. neoformans* var. *gattii*, profile VGII, in WA is indicative of the existence of an environmental source other than *E. camaldulensis* and *E. tereticornis*, since all

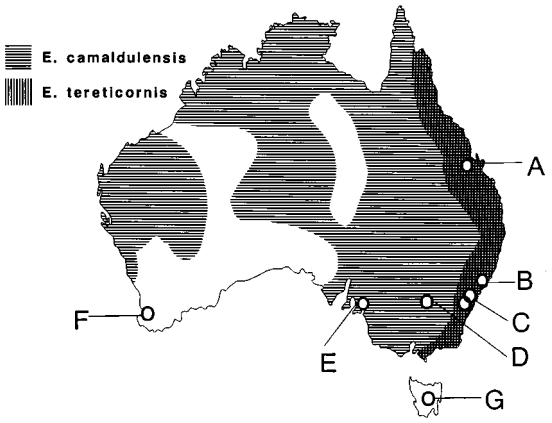

FIG. 1. Distribution of animals with cryptococcosis and natural habitats of *E. camaldulensis* and *E. tereticornis* in Australia. A, Currumbin sanctuary, Queensland (QLD); B, Port Macquarie, NSW; C, Sydney metropolitan area and Camden, NSW; D, West Wyalong, NSW; E, Adelaide, South Australia; F, Busselton, south of Perth, WA; G, Tasmania.

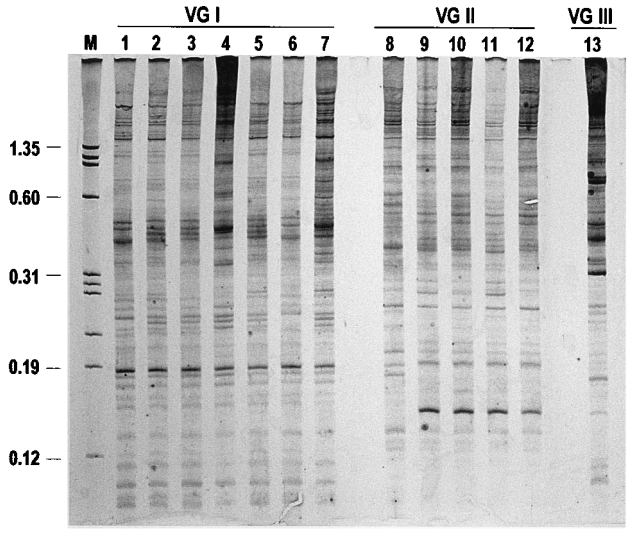

FIG. 2. RAPD profiles VGI, VGII, and VGIII from environmental and animal isolates of *C. neoformans* var. *gattii*. The primer pair used was MYC1-5SOR. Lane M, molecular size markers (in kilobases); lane 1, environmental isolate from California; lane 2, *E. camaldulensis* isolate; lane 3, *E. tereticornis* isolate; lane 4, horse isolate, WA; lanes 5 and 6, cat and dog isolates, respectively, NSW; lane 7, koala isolate, QLD; lanes 8 and 9, environmental isolates, California and WA; lane 10, horse isolate, WA; lane 11, cat isolate, NSW; lane 12, sheep isolate, WA; lane 13, environmental isolate, California. Size markers (lane M), phage ϕX174 DNA digested with the restriction enzyme *Hae*III. Note the identity of RAPD VGII patterns in all lanes except lane 8. The RAPD profile of the Californian environmental isolate in lane 8 is considered a variant of VGII (VGIIA) because its RAPD patterns are identical to those of the reference profile VGII with four primer pairs and different with three primer pairs (MYC1-5SOR and 5SOR-CN1 shown in Fig. 2 and 3 and FPK1-05–FPK1-07 [data not shown]).

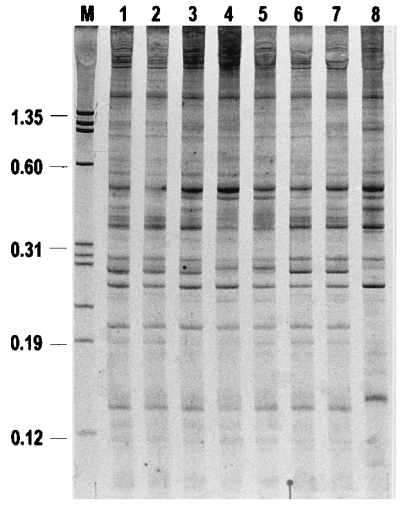

FIG. 3. RAPD profile VGII among animal and environmental isolates using primer pair 5SOR-CN1 (see Materials and Methods). Lane M, molecular size markers (in kilobases); lanes 1 and 2, cat isolates, NSW; lane 3, horse isolate, WA; lanes 4 to 6, sheep isolates, WA; lane 7, plant debris, WA; lane 8, environmental isolate, California. Size markers (lane M) are as described in the legend to Fig. 2.

Australian isolates from these eucalypts have been of profile VGI (16). Neither eucalypt is found naturally in the WA localities from which the sheep cryptococcal strains were obtained, although a different species of the red gum group, *Eucalyptus rudis*, occurs naturally in this area (2). *C. neoformans* var. *gattii*, profile VGII, was isolated from a horse in WA more than 3 years before the ovine infections occurred, suggesting that this genetic type is established in the area. The only environmental isolate of *C. neoformans* var. *gattii* from WA in our collection exhibited RAPD profile VGII and originated from plant material from the paddock housing the diseased sheep. It is uncertain whether the plant material had been contaminated with *C. neoformans* var. *gattii* from the respiratory secretions of these sheep or represented an environmental niche of the VGII strain. Isolates from two cats in NSW were also assigned to profile VGII. Neither cat had ventured outside the greater metropolitan area of Sydney, suggesting that the unknown environmental source of *C. neoformans* var. *gattii* profile VGII exists in NSW as well as WA, though probably less commonly.

Although the alternative environmental niche of *C. neoformans* var. *gattii* has not been established in this study, candidate trees include species of red gum other than *E. camaldulensis* and *E. tereticornis*. In WA and NSW, respectively, *E. rudis* and *E. amplifolia* subsp. *amplifolia* are native to the locations of the animals infected with *C. neoformans* var. *gattii* profile VGII, and in NSW, a second species, *Eucalyptus dealbata*, occurs nearby (2). We have conducted preliminary searches of debris and bark of *E. amplifolia* trees in an outer urban area of Sydney during their flowering season and in autumn (periods when collections from *E. camaldulensis* and *E. tereticornis* have been positive for *C. neoformans* var. *gattii* [5]), without success. Further investigation of the range of ecological niches of *C. neoformans* var. *gattii* may assist our understanding of the epidemiology of this significant human pathogen.

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REFERENCES

1. **Chen, S. C. A., A. G. Brownlee, T. C. Sorrell, P. Ruma, D. H. Ellis, T. Pfeiffer, B. R. Speed, and G. Nimmo.** Identification by random amplification of polymorphic DNA of a common molecular type of *Cryptococcus neoformans* var. *neoformans* in patients with AIDS or other immunosuppressive conditions. J. Infect. Dis., in press.
2. **Chippendale, G. M.** 1988. Flora of Australia, vol. 19. Bureau of Flora and Fauna. Australian Government Publishing Service, Canberra, Australia.
3. **Currie, B., D. Fisher, and D. H. Ellis.** Personal communication.
4. **Ellis, D. H.** 1987. *Cryptococcus neoformans* var. *gattii* in Australia. J. Clin. Microbiol. **25:**430–431.
5. **Ellis, D. H., and T. J. Pfeiffer.** 1990. Ecology, life cycle, and infectious propagule of *Cryptococcus neoformans*. Lancet **336:**923–925.
6. **Fisher, D., J. Burrow, D. Lo, and B. Currie.** 1993. *Cryptococcus neoformans* in tropical northern Australia; predominantly variant *gattii* with good outcomes. Aust. N. Z. J. Med. **23:**678–682.
7. **Kwon-Chung, K. J., and J. E. Bennett.** 1984. Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. Am. J. Epidemiol. **120:**123–130.
8. **Kwon-Chung, K. J., I. Polacheck, and J. E. Bennett.** 1982. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). J. Clin. Microbiol. **15:**535–537.
9. **Laurenson, I. F., S. Naraqi, A. Trevett, N. Howcroft, I. Burrows, and S. Saulei.** 1993. Cryptococcal meningitis in Papua New Guinea: ecology and the role of eucalypts. Med. J. Aust. **158:**213. (Letter.)
10. **Ley, C.** Personal communication.
11. **Malik, R., E. Dill-Macky, P. Martin, D. I. Wigney, D. B. Muir, and D. N. Love.** 1995. Cryptococcosis in dogs: a retrospective study of 20 consecutive cases. J. Med. Vet. Mycol. **33:**291–297.
12. **Malik, R., D. I. Wigney, D. B. Muir, D. J. Gregory, and D. N. Love.** 1992. Cryptococcosis in cats: clinical and mycological assessment of 29 cases and evaluation of treatment using orally administered fluconazole. J. Med. Vet. Mycol. **30:**133–144.
13. **Mitchell, D. H., T. C. Sorrell, A. M. Allworth, C. H. Heath, A. R. McGregor, K. Papanoum, M. J. Richards, and T. Gottlieb.** 1995. Cryptococcal disease of the CNS in immunocompetent hosts: influence of cryptococcal variety on clinical manifestations and outcome. Clin. Infect. Dis. **20:** 611–616.
14. **Pfeiffer, T. J., and D. H. Ellis.** 1992. Environmental isolation of *Cryptococcus neoformans* var. *gattii* from *Eucalyptus tereticornis*. J. Med. Vet. Mycol. **30:** 407–408.
15. **Riley, C. B., J. R. Bolton, J. N. Mills, and J. B. Thomas.** 1992. Cryptococcosis in seven horses. Aust. Vet. J. **69:**135–139.
16. **Sorrell, T. C., S. C. A. Chen, P. Ruma, W. Meyer, T. J. Pfeiffer, D. H. Ellis, and A. G. Brownlee.** 1996. Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA analysis and PCR fingerprinting. J. Clin. Microbiol. **34:**1253–1260.
17. **Spencer, A., C. Ley, P. Canfield, P. Martin, and R. Perry.** 1993. Meningoencephalitis in a koala (*Phascolarctos cinereus*) due to *Cryptococcus neoformans* var. *gattii* infection. J. Zoo Wild. Med. **24:**519–522.