DISTRIBUTION OF *CRYPTOCOCCUS NEOFORMANS* VAR. *GATTII* AMONG THE SPECIES OF *EUCALYPTUS*

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The distribution of cryptococcosis due to *Cryptococcus neoformans* var. *gattii* is geographically restricted, non-immunocompromised hosts are usually affected, large mass lesions in lung and/or brain (cryptococcomas) are characteristic and morbidity from neurological disease is high (Mitchell *et al.* 1995). Human disease is endemic in Australia (18 life threatening cases reported in 1995), Papua New Guinea, parts of Africa, India, South-East Asia, Mexico, Brazil, Paraguay and southern California (Ellis & Pfeiffer 1990; Kwon-Chung & Bennett 1984).

Environmental isolations, initially from the Barossa Valley in South Australia, have established that *C. neoformans* var. *gattii* has a specific ecological association with *Eucalyptus camaldulensis* Dehnh. (river red gum) and *Eucalyptus tereticornis* J.E. Smith (forest red gum) (Ellis & Pfeiffer 1990; Pfeiffer & Ellis 1992). Evidence for an epidemiological association between this cryptococcal habitat and human infection is circumstantial. There is correlation between the global distribution of human infection with *C. neoformans* var. *gattii* and the two species of eucalypts, and environmental searches conducted in Australia and elsewhere have so far failed to identify any other natural source. In Australia, *E. camaldulensis* is widespread, principally west of the Great Dividing Range, with endemic foci occurring around Darwin, Alice Springs, and rural areas of South Australia, New South Wales, Victoria, Queensland and Western Australia. *Eucalyptus tereticornis* shows a more restricted distribution occurring along the eastern coastal seaboard of Australia, extending to Papua New Guinea. It does not occur naturally in South Australia where our initial environmental sampling was performed. *Eucalyptus tereticornis* is easily confused with *E. camaldulensis* as they are morphologically similar, both belonging to the red gum group (*Eucalyptus* ser. *Exsertae* Blakely). These two eucalypts have been exported extensively from Australia to other regions; especially California, Mexico, Brazil, parts of Africa and South-East Asia (Ellis & Pfeiffer 1990) from where *C. neoformans* var. *gattii* infections have been reported (Kwon-Chung & Bennett 1984). Environmental isolates of the fungus have also been recovered from *E. camaldulensis* trees growing at a site near Fort Point, San Francisco, California and from *Eucalyptus* species growing in the car parks of the San Diego Zoo (Pfeiffer & Ellis 1992). To date, all *C. neoformans* var. *gattii* environmental isolates from eucalypts have been serotype B. *Cryptococcus neoformans* var. *gattii* serotype C has not yet been isolated from the environment.

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 per cent of clinical isolates (n = 48), exhibit a single major RAPD profile, designated VG1 (Sorrell & Chen *et al.* 1996). This genetic concordance between the majority of clinical and environmental isolates in Australia is consistent with the hypothesis that human disease is acquired from exposure to host eucalypts. Fingerprints of clinical isolates were independent of underlying host disease or body site of infection and profiles of all isolates were stable over time. Analysis by PCR-fingerprinting confirmed the RAPD results (Sorrell & Chen *et al.* 1996). However, a second RAPD profile (VGII) was found to be associated with human and animal infections in the southwest of Western Australia, where the two known host eucalypts do not occur naturally (Sorrell *et al.* 1996). This finding together with other reports of human and animal infection occurring in areas where neither *E. camaldulensis* or *E. tereticornis* are found raises the possibility of additional natural hosts for *C. neoformans* var. *gattii* (Sorrell & Chen *et al.* 1996; Sorrell & Brownlee *et al.* 1996). Accordingly, we have recently investigated eucalypt trees growing near Bunbury and Nannup in the southwestern corner of Western Australia and isolated *C. neoformans* var. *gattii* from woody debris from both *Eucalyptus rudis* Endl. and *Eucalyptus gomphocephala* DC.

Eucalyptus rudis (flooded gum) was deliberately targeted because it is a member of the red gum group with a very similar habitat to *E. camaldulensis* and it was endemic to the region under investigation. There are no records to indicate that *E. rudis* has been exported in any quantity to other countries. *Eucalyptus gomphocephala* (tuart) is a species, with no known close relatives, restricted to the subcoastal plains around Perth. This species has been extensively exported to California, Chile and the Mediterranean region especially Morocco, Cyprus, Algeria, Libya, Tunisia, Italy, Spain, Portugal, Greece, Malta and Israel. *Eucalyptus gomphocephala* was planted outside Australia for its quality hard wood which has tested to be stronger and tougher than oak, and earlier it was in great

demand for shipbuilding and underwater uses. However, preliminary RAPD analysis of the *C. neoformans* var. *gattii* isolates recovered from these two eucalypts demonstrate the common eucalypt VGI profile and not the expected VGII profile as seen in some clinical isolates from this area. The only environmental isolates so far recovered with a VGII profile are from plant debris collected from along the fence-line of a paddock containing sheep infected with the same biotype in southwestern Western Australia (Sorrell & Brownlee *et al.* 1996) and from debris collected from a possible hybrid of *E. camaldulensis* growing in California, USA (Sorrell & Chen *et al.* 1996). Further investigation of the range of ecological niches of *C. neoformans* var. *gattii* is warranted and we are now examining other closely related species and/or subspecies of eucalypts as natural reservoirs for the fungus. In addition, molecular characterisation of environmental and relevant clinical isolates has proved to be a valuable epidemiological tool, enabling the identification of subpopulations of *C. neoformans* var. *gattii* that may lead to the discovery of alternative host plants.

The following notes are provided to assist investigators wishing to isolate and identify *C. neoformans* var. *gattii* from host *Eucalyptus* or other trees. By far the best material to collect is decaying woody debris found accumulated around the base of suspect trees. Woody material from any small hollows, representing a sheltered habitat, should be especially sought after and sampled. Wood has previously been reported as a natural habitat for *C. neoformans* (Swinne *et al.* 1991) and our own data, from extensive collections, indicates that decaying eucalypt wood may be the principal source of *C. neoformans* var. *gattii* in nature. Wood debris from eucalypts contains very high concentrations of lignin and polyphenols which suggests that the well documented phenol oxidase activity of *C. neoformans* may be an adaptation to its natural habitat.

Identification of the trees is also important; so far positive samples have only been collected from *Eucalyptus camaldulensis*, *E. tereticornis*, *E. rudis* and *E. gomphocephala*, but other members of the red gum group (*Eucalyptus* ser. *Exsertae*) or hybrids may be involved. The identification of eucalypts growing outside Australia is also difficult and will likely require expert botanical knowledge. It should also be noted that the presence of *C. neoformans* var. *gattii* may be seasonal and that not all trees will be positive. Extensive sampling over a period of time is likely to be required.

When collecting specimens use small sealable plastic bags and collect a good size sample (at least a large hand full), label and return to the laboratory for processing. Specimens should be processed promptly, but if this is not possible store them in a cool environment away from direct sunlight. It should also be noted that delayed processing of damp or moist samples may lead to increased contamination.

For specimens consisting mostly of soil, take a small 'teaspoon' sized sample from the bulk collection and add 20 ml sterile distilled water and shake thoroughly. For specimens consisting mostly of leaf or woody material add 20–50 ml sterile distilled water to the sample and shake thoroughly. Larger pieces of wood may have to be picked out and processed individually. Allow washings to stand for 5–10 minutes and then plate out by streaking 0.5–1.0 ml aliquots onto bird seed agar (use smaller aliquots if contamination is a problem). Incubate plates at 26°C.

In our experience the best primary isolation medium to use is Staib's recipe for bird seed agar. Many different formulations for bird seed agar or other selective agars for the isolation of *Cryptococcus neoformans* have been published, but they are not as good as that documented by Staib (1987).

The initial or early recognition of colonies of *C. neoformans* growing on the bird seed agar is a technique which requires considerable experience and expertise. Plates must be examined daily and maintained for seven days. Look for small brown pigmented 'pin head' colonies. These usually appear after 2–3 days of incubation and are mucoid in appearance. Isolates of *C. neoformans* var. *gattii* are generally more mucoid than those of *C. neoformans* var. *neoformans*. Holding the primary isolation plates up to direct sunlight may also help to see the small brown colonies of *C. neoformans*. However, we must stress again that careful examination of the plates is required, colonies of *C. neoformans* are easily missed, especially if there are many other contaminant fungi growing on the plate.

Suspect colonies should be picked off and subcultured by streaking for purity on to bird seed agar. It is essential that all isolates are fully identified by using one of the recognised yeast identification schemes utilising sugar assimilation tests (*e.g.* reliable commercially available yeast identification kits are the API 20C, ID 32C, Uni-Yeast-Tek, MicroScan or Vitek systems).

Some contaminant fungi on initial appearance may look suspiciously like *C. neoformans*, however, once streaked for purity they show hyphal development and are clearly moulds. In addition, other yeasts are often isolated from eucalypt material, especially strains of *Cryptococcus laurentii* which may also show a brown colour effect on bird seed agar and turn CGB media blue.

Varietal differentiation should be done using CGB agar (Kwon-Chung *et al.* 1982). This simple biotype test is based on the ability of *C. neoformans* var. *gattii* isolates to grow in the presence of L-canavanine and to assimilate glycine as a sole carbon source. *Cryptococcus neoformans* var. *gattii* isolates usually turn CGB agar blue within 3–5 days, however, some strains may take as long as 10–14 days.

Key features for the identification of *Cryptococcus neoformans*

Microscopic morphology, physiological and biochemical tests, pigmentation on bird seed agar and reaction on Canavanine-glycine-bromthymol blue agar.

On Sabouraud's dextrose agar colonies are cream colored, smooth, mucoid and yeast-like, consisting of globose to ovoid budding yeast-like cells or blastoconidia, 3.0–7.0 × 3.3–7.9 µm. India ink preparations show the presence of distinct, wide gelatinous capsules surrounding the yeast cells.

Physiological Tests:	
Germ tube test	Negative
Hydrolysis of urea	Positive
Growth on cycloheximide agar	Negative
Growth at 37°C	Positive (w)

Assimilation Tests:		**Potassium nitrate**	-		
Glucose	+	Lactose	-	Ribitol	v
Galactose	+	Raffinose	+(w)	L-Sorbose	v
Maltose	+	Cellobiose	+(w)	D-Ribose	+(w)
Sucrose	+	Melibiose	-	Galactitol	+(D)
Trehalose	+	Erythritol	-	D-Mannitol	+
D-Xylose	+	Inositol	+	D-Glucitol	+
Soluble Starch	v	L-Rhamnose	+	Salicin	v
Melezitose	+	D-Arabinose	+	Citric acid	v
Glycerol	v	L-Arabinose	+(D)	DL-Lactic acid	v
Succinic acid	v				

+ Positive, - Negative, v Variable, w Weak, D Delayed.

Bird Seed Agar for the selective isolation of *Cryptococcus neoformans* (Staib 1987)	
Guizotia abyssinica (niger seed)	50 g
Glucose	1 g
KH_2PO_4 (potassium dihydrogen orthophosphate)	1 g
Creatinine	1 g
Bacto-agar (Difco)	15 g
Distilled water	1000 ml

Additives: to each 500 ml bottle.

Penicillin G (20 units/ml)	0.5 ml
Gentamicin (40 mg/ml)	0.5 ml

1. Grind seeds of *Guizotia abyssinica* as finely as possible with an electric mixer and add to 1000 ml distilled water in a stainless steel jug.
2. Boil for 30 minutes, pass through filter paper and adjust volume to 1000 ml.
3. Add remaining ingredients except Bacto-Agar to filtrate and dissolve.
   If required: Cool to room temperature and pH to 5.5.
   Dispense into 500 ml bottles.
4. Add 7.5 g Bacto-agar to each 500 ml reagent bottle.
5. Autoclave at 110°C for 20 minutes.
6. Cool to 48°C and add 0.5 ml Penicillin G and 0.5 ml Gentamicin to each 500 ml of Bird Seed Agar.
7. Mix gently and pour into 90 mm plastic petri dishes.

CGB (L-Canavanine, glycine, bromthymol blue) agar for the differentiation of *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *gattii* (Kwong-Chung *et al.* 1982).

Solution A	
Glycine	10 g
KH_2PO_4	1 g
$MGSO_4$	1 g
Thiamine HCl	1 mg
L-canavanine sulphate	30 mg
Distilled water	100 ml

1. Dissolve ingredients in small beaker and adjust pH to 5.6.
2. Filter sterilise solution using 0.22 °µm filter.
3. Store in refrigerator.

Solution B (Aqueous Bromthymol Blue)	
Bromthymol blue	0.4 g
0.01N NaOH	64 ml
Distilled water	36 ml

1. Dissolve the Bromthymol Blue in the NaOH.
2. Add the water to this.

To prepare medium (1L for plates)	
Distilled water	880 ml
Solution B	20 ml
Bacto agar	20 g

1. Autoclave to 121°C for 15 minutes, cool to 48°C.
2. For plates add 100 ml of the filtered solution A and mix.
3. Dispense in plates.

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1996 COMMEMORATIVE CONFERENCE
University of Melbourne, October 1996
PROVISIONAL PROGRAM
MYCOLOGY

Wednesday, 3rd October
8.00–8.50 Registration / Set up posters

Session 1 (Chairperson: Jack Simpson, President, Australasian Mycological Society)
8.50 Welcome
9.00 Ethel Irene McLennan, an Australian mycological pioneer—*Sophie Ducker*
9.30 Mycological studies on small Victorian fungi by Harry Swart and Gordon Beaton—*Gretna Weste*
9.50 Mushroom poisonings in Australia—*F. Mary Cole*
10.10 Observations on morphology and the response of hyphae to temperature by Australian and French isolates of *Lepista*—*Karen Stott, Andrew Broderick & N.G. Nair*

10.30 Morning Tea

Session 2 (Chairperson: Cheryl Grgurinovic)
10.50 Coprophilous fungi in New Zealand with special emphasis on the genus *Podospora* (Lasiosphaeriaceae)—*Ann Bell & Daniel Mahoney*
11.10 The species of *Elsinoë* on Myrtaceae in Australia—*Jack Simpson*
11.30 *Lactarius* (Russulaceae) in Tasmania: with special reference to *L. subdulcis*—*Naiyana Thongjeim & Alan Mills*
11.50 A study of the genus *Amanita* (Agaricales)—*Alec Wood*
12.10 Preliminary observations on the systematics of the Australian Hygrophoraceae Lotsy (Fungi, Agaricales)—*Anthony Young*

12.30 **Launch of *Fungi of Australia*** (Senator Hill)

12.50 Lunch

1.30 Poster Session