Review

Cryptococcosis and the Ecology of *Cryptococcus neoformans*

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Abstract

Cryptococcosis is a cosmopolitan infectious disease of humans and animals caused by an encapsulated basidiomycetous yeast-like fungus *Cryptococcus neoformans*. Clinical manifestations range from asymptomatic carriage in the respiratory tract to chronic pulmonary disease to meningitic disease with dissemination to the skin and other sites also reported. The incidence of infections varies with geographic location and with patient groups; AIDS has now become the most common predisposing factor for infection. Cryptococcal virulence factors include the ability to form a polysaccharide capsule, produce melanin and to grow at 37°C. *C. neoformans* var. *neoformans* (serotypes A and D) has a world-wide distribution and been isolated from various sources in nature and is noted for its association with accumulations of avian guano, especially with pigeon excreta. *C. neoformans* var. *gattii* (serotypes B and C) has a more restricted global distribution corresponding to a subtropical to tropical climate. Environmental isolations have now established that *C. neoformans* var. *gattii* serotype B appears to have a specific ecological association with *Eucalyptus camaldulensis* and *Eucalyptus tereticornis*. The global distribution of these two trees appears to correspond to the epidemiologic distribution of cryptococcosis caused by *C. neoformans* var. *gattii*. The epidemiology of cryptococcosis can primarily be explained by exposure to an infective aerosolized inoculum, such as basidiospores released from specific host plants and/or desiccated blastoconidia (yeast cells) disseminated from accumulations of dried pigeon dung. The ecology of *C. neoformans* still remains largely unresolved, studies on the host-parasite interaction between serotype B and *E. camaldulensis* are still in progress, and extensive environmental searches are now underway to determine the natural habitats of serotypes A, C and D.

Key words: Cryptococcosis, *Cryptococcus neoformans*, Ecology, *Eucalyptus*, Epidemiology

Introduction

Cryptococcosis is a cosmopolitan infectious disease of humans and animals caused by an encapsulated basidiomycetous yeast-like fungus called *Cryptococcus neoformans* (Fig 1). The polysaccharide capsule produced by this fungus

exhibits antigenic variability, and four serotypes (A, B, C and D) can be distinguished by agglutination of the organism with specific antisera¹⁻²⁾. Important differences between the AD and BC serotype pairs have been demonstrated and *C. neoformans* has been subdivided into two varieties: *Cryptococcus neoformans*

Fig. 1. Encapsulated yeast cell of *C. neoformans* var. *gattii* ($\times 1750$).

var. *neoformans* (serotypes A, D and AD) and *Cryptococcus neoformans* var. *gattii* (serotypes B, C and BC)³⁾.

Mating experiments have shown that the two varieties of *C. neoformans* exhibit different sexual states: *Filobasidiella neoformans* var. *neoformans* is the teleomorph of *C. neoformans* var. *neoformans* while *Filobasidiella neoformans* var. *bacillispora* is the teleomorph of *C. neoformans* var. *gattii*^{3,4)}. The teleomorph is the sexual state of a fungus, which in this case is characterized by the production of basidiospores (sexual propagules). Asexual reproduction in *C. neoformans* is the anamorph state which is characterized by the production of budding yeast-like cells (asexual propagules) that typically develop a wide polysaccharide capsule.

Clinical Manifestations and Major Predisposing Factors

Cryptococcosis is a chronic, subacute to acute pulmonary, systemic or meningitic disease, initiated by the inhalation of basidiospores and

/or desiccated yeast cells of *C. neoformans*. Primary pulmonary infections have no diagnostic symptoms and are usually subclinical. On dissemination, the fungus usually shows a predilection for the central nervous system, however skin, bones and other visceral organs may also become involved. Although *Cryptococcus neoformans* is regarded as the principle pathogenic species, *C. albidus* and *C. laurentii* have on occasion also been implicated in human infection⁵⁾.

The true incidence of cryptococcosis is difficult to estimate as the disease is generally not notifiable. However, it has been described as the "sleeping giant" among infections caused by fungi and many thousands of individuals are probably infected every year⁶⁾. Two main groups of patients may be recognized, those with AIDS and those without AIDS.

In the non-AIDS group, a large percentage of patients present with no obvious predisposing conditions, they are otherwise healthy individuals. The remainder are often patients with deficiencies in cell-mediated immunity due to

corticosteroid therapy, sarcoidosis, lymphoma, organ transplantation and collagen vascular disease. The incidence of cryptococcosis in non-AIDS patients has been estimated to be 2 cases per million population per year⁷⁾.

AIDS associated cryptococcosis usually occurs in HIV patients when their CD4 lymphocyte count is below 200/mm³ and as such is one of the "AIDS defining illnesses". Meningitis is the predominant clinical presentation with fever and headache as the most common symptoms⁸⁻⁹⁾. AIDS has now become the most common detectable predisposing factor for cryptococcosis. The incidence of cryptococcosis in AIDS patients varies from 3 to 30% [13% in Australia] and is dependent on geographic location.

Cryptococcal virulence factors include the presence of a polysaccharide capsule, the ability to produce melanin due to phenoloxidase activity and growth at 37°C⁸⁾. Acapsular mutants of *C. neoformans* are less pathogenic and are readily ingested by leukocytes *in vitro*. However, it appears that the degree of encapsulation is not related to virulence. Similarly, melanin negative mutants of *C. neoformans* are avirulent in mice. The ability of *C. neoformans* to produce melanin when grown on certain media, such as Bird seed agar, has also proved to be a useful phenotypic marker of the presumptive identification of the fungus. Finally, mutants of *C. neoformans* unable to grow at 37°C are also avirulent regardless of their ability to form capsules or to form melanin⁸⁾. It is also of interest that clinical isolates of *C. neoformans* var. *gattii* have a slower growth rate than environmental isolates originating from *Eucalyptus camaldulensis*. These eucalypt isolates readily produce fatal infections in mice, whereas the slower growing clinical isolates do not.

Important differences in virulence have also been observed between the two varieties of *C. neoformans*. *C. neoformans* var. *neoformans* appears to be more virulent, it elicits a weak host response and predominantly causes infections in immunosuppressed patients. These

infections generally respond well to therapy, however mortality may be high. On the other hand, *C. neoformans* var. *gattii* appears to be less virulent, it elicits a strong host response and causes infections mostly in normal hosts. These infections tend to be more refractory to therapy and treatment times may be longer, however mortality is generally low¹⁰⁾.

Pulmonary Cryptococcosis

Asymptomatic carriage of *C. neoformans* has been reported from the respiratory tract, especially sputum and from skin in healthy people as a result of normal environmental exposure¹¹⁾. In addition, patients with chronic lung disease, such as bronchitis and bronchiectasis, may also have asymptomatic colonization, with *C. neoformans* being isolated from their sputum over many years⁹⁾. Subclinical cryptococcosis may result from environmental exposure, with normal individuals experiencing a self-limiting pneumonia with accompanying sensitization. Most primary infections of this type have no diagnostic symptoms and are usually discovered only by routine chest x-ray^{5,8)}. When present, symptoms include cough, low-grade fever and pleuritic pain. Invasive pulmonary cryptococcosis may occur in some patients when primary infections do not readily resolve, leading to a more chronic pneumonia progressing slowly over several years. Patients may become pyrexemic and have an accompanying cough, however many pulmonary lesions are often asymptomatic, especially when chronic granulomas are formed. Chronic pulmonary cryptococcosis also increases the risk of dissemination to the central nervous system^{5,8,9,12-15)}.

Central Nervous System

Dissemination to the brain and meninges is the most common clinical manifestation of cryptococcosis and includes meningitis, meningoencephalitis or expanding cryptococcoma^{5,8,9)}. Meningitis is the most common clinical form, accounting for up to 85% of the total number of cases, however the clinical signs

are rarely dramatic. Symptoms usually develop slowly over several months, and initially include headache, followed by drowsiness, dizziness, irritability, confusion, nausea, vomiting, neck stiffness and focal neurological defects, such as ataxia. Diminishing visual acuity and coma may also occur in later stages of the infection. Acute onset cases may also occur, especially in patients with widespread disease, and these patients may deteriorate rapidly and die in a matter of weeks. Meningoencephalitis due to invasion of the cerebral cortex, brain stem and cerebellum is an uncommon, rapid fulminate infection, often leading to coma and death within a short time. Symptoms include slow response to treatment and signs of cerebral edema or hydrocephalitis, especially papilledema. Cryptococcoma is a rare entity, characterized by localized, solid, tumorlike masses, usually found in the cerebral hemispheres or cerebellum, or more rarely in the spinal cord. Symptoms are consistent with an expanding intracranial mass and include headache, drowsiness, nausea, vomiting, mental changes, slurred speech, double vision, unsteadiness of gait, coma, paralysis and hemiparesis. These symptoms may mimic cerebral neoplasm which may delay a true diagnosis^{5, 8, 9, 16-19}).

Cutaneous Cryptococcosis

Primary cutaneous cryptococcosis in the form of ulcerated lesions or cellulitis occasionally occurs, especially in immunosuppressed patients. These lesions may resolve spontaneously or with systemic antifungal treatment. However, all patients with skin lesions should be monitored carefully for possible dissemination to the central nervous system. Secondary cutaneous infections occur in up to 15% of patients with disseminated cryptococcosis and often indicate a poor prognosis. Lesions usually begin as small papules that subsequently ulcerate, but may also present as abscesses, erythematous nodules, or cellulitis. In patients with AIDS, skin manifestations represent the second most common site of disseminated cryptococcosis. Lesions often occur on the head and neck

and may present as papules, nodules, plaques, ulcers, abscesses, cutaneous ulcerated plaques, herpetiform lesions, lesions simulating both molluscum contagiosum and Kaposi's sarcoma. Anal ulceration may also occur^{5, 8, 9, 20-22}).

Cryptococcosis of Bone

Osseous cryptococcosis occurs in up to 10% of disseminated cases and may involve bony prominences, cranial bones and vertebrae. The lesions are lytic without periosteal reaction and symptoms of dull pain on movement are reported. Occasional cases of arthritis have also been reported, mostly involving the knee joint^{5, 8, 9, 23}).

Ocular Cryptococcosis

Ocular manifestations of Cryptococcosis most commonly include papilledema and optic atrophy, due to raised intracranial pressure. Other ocular signs of cryptococcosis are uncommon and usually occur as a result of dissemination^{5, 8, 9, 24, 25}).

Other forms of Cryptococcosis

Cryptococcus neoformans is often isolated from urine of patients with disseminated infection. Occasionally, signs of pyelonephritis or prostatitis may be observed. Other rare forms of cryptococcosis include adrenal cortical lesions, endocarditis, hepatitis, sinusitis, and localized oesophageal lesions^{5, 8, 9, 26, 27}).

Laboratory Diagnosis, Environmental Isolation and Identification

The demonstration of encapsulated yeast cells in CSF, biopsy tissue, blood or urine by direct microscopy should be considered significant, even in the absence of clinical symptoms. Positive sputum specimens should be considered potentially significant, even though *C. neoformans* may also occur in respiratory secretions as a saprophyte. Basically, all patients with a positive microscopy for cryptococci, from any site should be investigated for disseminated disease, especially by culture and antigen detection^{5, 8, 28}). Positive culture of CSF is definitive.

However, positive culture of respiratory secretions, especially in patients without clinical symptoms, needs to be interpreted with some caution, until additional supporting evidence is available. Isolation of *Cryptococcus albidus* or *C. laurentii*, should also be interpreted with caution as these species are infrequent pathogens and once again, additional supporting clinical and microscopic evidence is necessary^{5, 9, 28}).

It should also be noted that the detection of cryptococcal capsular polysaccharide antigen in spinal fluid is now the method of choice for diagnosing patients with cryptococcal meningitis. In AIDS patients, cryptococcal antigen can be detected in the serum in nearly 100% of cases. However, in non-AIDS patients antigen detection in serum is less sensitive, with only about 60% of patients with cryptococcosis reported as positive. Note, serum specimens should be pretreated with pronase to enhance detection of antigen and avoid false negative results⁹).

Environmental samples, including soil, guano and vegetation, such as wood, bark, leaves, and accumulated organic debris were collected in small sterile plastic bags. These samples were shaken in 20 ml of sterile distilled water and allowed to stand for 10 to 15 minutes, and then 2.0 ml aliquots of the resulting suspensions were streaked onto Staib's Bird Seed (*Guizotia abyssinica*) agar²⁹). Cultures were examined daily, and all yeast colonies exhibiting a brown colour pigmentation were subcultured and identified.

In our laboratory, the identity of all environmental and clinical isolates was confirmed using a battery of physiological, biochemical and morphological tests, including fermentation and assimilation studies based on those used at the Centraalbureau voor Schimmelcultures, Delft, The Netherlands³⁰). Reliable yeast identification kits are available commercially, such as the API 20C AUX, ATB 32C, Micro-Scan and Vitek systems may also be used to identify this yeast. Please note that it is important to start with a pure culture by

streaking for single colonies.

Canavanine-glycine-bromthymol blue (CGB) agar is now the media of choice to determine the varietal status of *C. neoformans* isolates³¹). 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.

Initial serotyping studies were performed using antisera raised by immunizing rabbits with formalin-killed *C. neoformans* cells, using a combination of previously published methodologies^{1, 32, 33}). Antisera used for final serotyping were first adsorbed with a mixture of cells of the other serotypes. Equal volumes of cells and antisera were tested by slide agglutination. The antigens used for the production of antisera were prepared from the following type strains of *Cryptococcus neoformans*, obtained from the American Type Culture Collection (ATCC): serotype A (ATCC 34871), serotype B (ATCC 34878), serotype C (ATCC 34880) and serotype D (ATCC 34875). More recently, we have been using the "Crypto Check" kit manufactured by Iatron Laboratories³⁴), which is a slide agglutination test using selected antisera against the five antigenic factors that are necessary for serotyping isolates of *C. neoformans*.

Ecology

C. neoformans var. *neoformans* (A, D and AD serotypes) has a world-wide distribution and is a major cause of all clinical types of cryptococcosis. It has been isolated from various sources in nature and is noted for its association with accumulations of avian guano, especially with pigeon excreta. The fungus has also been isolated from the dung of caged birds including canaries, parrots and budgerigars. Other environmental isolations of *C. neoformans* var. *neoformans* have been from rotting vegetables, fruits and fruit juices, wood, dairy products and soil^{5, 35, 36}).

On the other hand, *C. neoformans* var. *gattii* (serotypes B and C) has a more restricted global distribution corresponding to a subtro-

pical to tropical climate³⁷⁾. It is also a major causative agent of all clinical types of cryptococcosis. An assessment of the available data from Australian patient isolates indicated that rural areas of South Australia and the Northern Territory were endemic regions for the occurrence of this pathogenic fungus³⁸⁾. Environmental isolations, initially from the Barossa Valley in South Australia³⁹⁾, have established that *C. neoformans* var. *gattii* has a specific ecological association with *Eucalyptus camaldulensis* (the river red gum)

(Fig 2). More recent environmental work has now also established an association between *C. neoformans* var. *gattii* and *Eucalyptus tereticornis* (the forest red gum)⁴⁰⁾.

Culture characteristics of these eucalypt isolates of *C. neoformans* var. *gattii* include the following: very mucoid growth on Sabouraud dextrose agar, intense brown colour effect on Staib's bird seed agar²⁹⁾, strong assimilation of creatinine, assimilation of D-proline, high virulence for white mice and a positive reaction on CGB media within 5 days. In addition,

Fig. 2. *E. camaldulensis* growing on the banks of the Murrumbidgee River at Balranald, positive for *C. neoformans* var. *gattii*.

30 environmental isolates of *C. neoformans* var. *gattii* were randomly selected for drug sensitivity testing and all were found to be fully sensitive to the following 6 antifungal drugs; Amphotericin B, Miconazole, Ketoconazole, 5-Fluorocytosine, Itraconazole and Flucanazole. Thus, it would appear that there is no inherent resistance associated with environmental isolates of this fungus.

The global distribution of both *E. camaldulensis* and *E. tereticornis* is similar and appears to correspond to the epidemiologic distribution of cryptococcosis caused by *C. neoformans* var. *gattii*^{39, 40}). 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 Australia⁴¹) (Fig 3). *E. tereticornis* shows a more restricted distribution occurring along the eastern coastal seaboard of Australia, extending to Papua New Guinea⁴¹) (Fig 4). It does not occur naturally in South Australia where our initial environmental sampling was performed.

E. tereticornis is often confused with *E. camaldulensis* as they are morphologically similar both belonging to the red gum group (*Eucalyptus* ser. *Exsertae* Blakely)⁴¹). The two species are so closely related that they are only distinguished by their operculum shape and size. *E. camaldulensis* has an operculum which is hemispherical, rostrate or conical, obtuse, 4-6 mm long, 3-6 mm wide whereas *E. tereticornis* has a conical operculum, 8-13 mm long, 4-6 mm wide⁴¹).

These two eucalypts have been exported extensively from Australia to other regions; especially California, Mexico, Brazil, parts of Africa and South East Asia⁴²) from where *C. neoformans* var. *gattii* infections have been reported³⁷). We have recovered environmental isolates of the fungus from *E. camaldulensis* trees growing at a site near Fort Point, San Francisco, California⁴³). More recent field work in California has yielded further environmental

isolates of *C. neoformans* var. *gattii* from *Eucalyptus* species growing in the car parks of the San Diego Zoo. Thus providing further evidence that this important medical pathogen has been exported from Australia along with the river and forest red gums.

Electrophoretic karyotype studies have now demonstrated similar chromosomal banding patterns in four strains of *C. neoformans* var. *gattii* recovered from *E. camaldulensis* trees growing in different geographic locations⁴⁴). Three of the isolates came from trees growing in Australia from sites at least 15 to 500 km apart, while the fourth isolate was recovered from a tree growing in San Francisco, California⁴⁴). More recently, a molecular comparison using randomly amplified polymorphic DNA markers (RAPD) has demonstrated that a clinical isolate of *C. neoformans* var. *gattii* was indistinguishable from 5 environmental isolates obtained from *E. camaldulensis* trees growing in the patients immediate home vicinity [personal communication Dr. A. Brownlee, CSIRO, Division of Animal Production, Sydney, Australia]. These results provide further evidence to support the direct relationship between *C. neoformans* var. *gattii* and *E. camaldulensis* and human clinical infections caused by this fungus.

To date, all *C. neoformans* var. *gattii* environmental isolates from both *E. camaldulensis* and *E. tereticornis* have been serotype B. Serotype data from 460 Australian clinical and environmental isolates of *Cryptococcus neoformans* yielded 167 isolates of serotype A, 274 isolates of serotype B, 2 isolates of serotype D and no serotype C isolates, while 17 isolates were classified as untypeable. These results show a similar pattern to other global data for serotypes A and B, and further highlight the low frequency of serotypes C and D²) (Table 1).

In South Australia, dispersal of infectious propagules of *C. neoformans* var. *gattii* appears to occur from November to March, concomitant with the flowering of *E. camaldulensis* and *E. tereticornis*³⁹). Direct microscopy of

Fig. 3. Distribution of *E. camaldulensis* in Australia⁴¹⁾.

Fig. 4. Distribution of *E. tereticornis* in Australia⁴¹⁾.

Table 1. Serotypes of Australian Clinical and Environmental isolates of *C. neoformans*

	A	B	C	D	UT	TOTAL
Clinical	95	98	0	1	9	203
Environmental	80	184	0	2	8	274
Total	175	282	0	3	17	477

Table 2. Relative size and shape of potential infectious propagules of *Cryptococcus neoformans*

	var. <i>neoformans</i>	var. <i>gattii</i>
Basidiospores	spherical, oblong or elliptical with finely rough walls 2-3×2-5 um	bacillary with smooth walls 1-2×3-8 um
Source	Unknown	<i>Eucalyptus</i> sp.
Yeast Cells	globose or oval 3-8 (15) um (less than 2 um when desiccated)	globose or oval 3-8 (15) um
Source	Avian Guano	Unknown ? <i>Eucalyptus</i> sp.

water suspensions⁴⁵⁾ that were strongly positive on culture for the fungus demonstrated the presence of numerous smooth-walled encapsulated bacillary-like structures (1.5-2×6-8 um), similar in shape and size to that described for basidiospores belonging to the teleomorph *F. neoformans* var. *bacillispora* (Table 2).

The significance of the difference in size of the potential infectious propagules of *C. neoformans* has been debated in the literature for some time⁴⁶⁻⁴⁸⁾. Many authors favour the desiccated yeast cell as the infectious unit for the following reasons (1) their small size, (2) the environmental source has been identified as pigeon dung and (3) compatible mating types are not usually found in the pigeon dung thus excluding the formation of basidiospores. However, in view of our findings with *C. neoformans* var. *gattii* it would appear that both yeast cells and basidiospores may act as infectious propagules, and that size differences may help explain some of the varietal differences in the virulence and/or pathogenicity of *C. neoformans*.

It would appear that basidiospores not landing on susceptible *E. camaldulensis* host

tissue synthesize protective capsular material. These remain dormant until they are either stimulated by a suitable growth medium to replicate as budding yeast cells, or cleared from the environment by the action of sunlight and other microorganisms. The transformation of basidiospores into encapsulated yeast cells is well documented³⁾. Although both are haploid cells, yeast cells are the only vegetative units that grow readily on artificial media. Further studies on the host-parasite interaction are in progress to determine where the formation of dikaryotic hyphal elements and basidia of the fungus occur in the host trees.

Presently we are investigating decaying eucalypt wood, as a natural habitat of *C. neoformans* var. *gattii*. Wood has previously been reported as a natural habitat for *C. neoformans*³⁶⁾ 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 *E. camaldulensis* is unique in that it contains up to 10 times the amount of lignin and polyphenols as wood from other eucalypts. It is of interest to note that phenol oxidase

enzyme activity is an essential requirement for lignin degradation, especially by some basidiomycetous fungi⁴⁹⁾. *Cryptococcus* has already been reported as an important genus in the degradation of cellulose and the delignification of wood⁵⁰⁾. We are studying the ability of *C. neoformans* var. *gattii* isolated from eucalypt wood for lignocellulose-degrading enzyme activity. Perhaps the well documented phenol oxidase activity of *C. neoformans* is an adaptation to its natural habitat, rather than to its accidental infection of humans and animals. We are also currently examining other closely related species and/or sub-species of red gums as natural reservoirs for *Cryptococcus neoformans* var. *gattii*. In addition, molecular characterization of environmental and relevant clinical isolates to assess more completely the natural host genotype/serotype relationships is also being undertaken.

Conclusion

In conclusion, we believe that *C. neoformans* is either an endophytic or epiphytic yeast-like fungus and that the epidemiology of cryptococcosis can primarily be explained by exposure to an infective aerosolized inoculum⁴⁵⁾. For *C. neoformans* var. *gattii* we have postulated that the principle infectious propagule is the basidiospore and that exposure to *E. camaldulensis*, *E. tereticornis* or other host eucalypt trees, is required to initiate infection in man and animals⁴⁵⁾. For *C. neoformans* var. *neoformans* we have postulated that both the basidiospore and desiccated encapsulated yeast cells act as infectious propagules. The basidiospores showing a seasonal distribution in association with an as yet unidentified host plant, while the latter are dispersed from accumulations of dried pigeon/bird dung which acts as a year round vector⁴⁵⁾. The need for exposure to flowering eucalypt trees in order to acquire an infection by *C. neoformans* var. *gattii* serotype B also provides an explanation for the high incidence of infections caused by this fungus in Australian Aborigines living in the Northern Territory³⁹⁾ and possibly for

it's low world-wide incidence in AIDS patients^{35, 51, 52)}.

Acknowledgements

We wish to thank Prof. Hiroshi Nakajima, Department of Dermatology, Yokohama City University for giving us the opportunity to present this paper at the 37th Annual Meeting of the Japanese Society for Medical Mycology.

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この論文は、第37回日本医真菌学会総会において招請講演として発表されたものです。