

## First Identification of Autochthonous *Cryptococcus neoformans* var. *gattii* Isolated from Goats with Predominantly Severe Pulmonary Disease in Spain

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*Cryptococcus neoformans* var. *gattii* is associated with *Eucalyptus* trees growing in various tropical and subtropical regions of the world. The identification of 13 autochthonous strains of *C. neoformans* var. *gattii* in Spain is reported. These strains were isolated from lung (10 samples), liver (1 sample), and brain (2 samples) tissue specimens from six goats suffering from predominantly severe pulmonary disease that were autopsied. The animals were members of five different herds of goats grazing in rural areas of the province of Cáceres (Extremadura, Spain). Between 1990 and 1994, there were five outbreaks, in which between 2.5 and 12% of the goats were affected. Although respiratory symptoms (pneumonia) associated with cachexia were the predominant clinical picture in all outbreaks, brain and liver involvement was also documented in three of the five outbreaks. Biotyping was performed by culturing the isolates on L-canavanine-glycine-bromothymol blue medium and testing them for the assimilation of D-proline and D-tryptophan. Serotyping by agglutination tests confirmed the characterization of all strains as *C. neoformans* var. *gattii* serotype B. This is the first confirmation of the presence of this variety in Spain, with a peculiar ability to produce severe pulmonary and systemic disease in normal goats, particularly in the form of outbreaks of pneumonia in association with cachexia.

*Cryptococcus neoformans* is a capsulated yeast with a worldwide distribution. Since its description at the end of the last century, it has been isolated from different substrata from nature and it has been shown that the basidiomycete *Filobasidiella neoformans* is its teleomorph (15). Humans and other animals are infected by inhalation, developing cryptococcosis, which is especially severe in immunocompromised individuals, in particular in those infected with the human immunodeficiency virus (4).

Two varieties of *C. neoformans* have been described, *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* (6). Each variety has its distinctive serotypes based on the antigenic composition of its capsular polysaccharides, which play an important role in pathogenicity. *C. neoformans* var. *neoformans* consists of serotypes A, D, and AD, whereas *C. neoformans* var. *gattii* has the B and C serotypes (12). Substantial differences in the ecology of the two varieties have been described and noted to influence the epidemiology of cryptococcosis (17).

*Cryptococcus neoformans* var. *neoformans* has a worldwide distribution. It is isolated frequently from the droppings of birds, mainly the fecal pigeon (*Columba livia*), since it can grow in substrates containing high concentrations of creatinine (28). This variety is responsible for the majority of the cases of cryptococcosis in immunocompromised patients (19). By contrast, *C. neoformans* var. *gattii* has not been isolated from bird droppings, apparently because it has a lower tolerance for high

levels of creatinine. Its optimum growth temperature is 32°C, and plant debris are its natural reservoir, especially those of *Eucalyptus camaldulensis* (9) and *Eucalyptus tereticornis* trees (25). More rarely, it has been isolated from bat feces, a wasp's nest, and other substrata (10, 18). The epidemiology of the infections produced by *C. neoformans* var. *gattii* is also different, with infections appearing to occur predominantly in tropical and subtropical areas of Australia, Brazil, Kenya, Zaire, Southeastern Asia, and Southern California, affecting people with no impairment of their immunological status (8).

Cryptococcosis in wild and domestic mammals, with sporadic cases in cats, dogs, goats, horses, and sheep, has also been reported (3). Outbreaks in bovine and caprine livestock have also been identified (1, 23). Information regarding the variety of *C. neoformans* that causes infection in animals is scarce (11). In Europe, isolation of *C. neoformans* var. *gattii* is exceptional (20, 21).

We describe the first identification of *C. neoformans* var. *gattii* in Spain. The organism was isolated from lung, liver, and brain tissue specimens of six infected goats from five different herds in which five outbreaks of severe pulmonary and systemic disease occurred from 1990 to 1994.

### MATERIALS AND METHODS

**Organisms.** Thirteen strains of *C. neoformans* isolated from tissue samples obtained by autopsy of six goats with subacute or chronic pneumonia associated with cachexia from farms in different districts were studied. These animals, of different *Capra hircus* races (*verata*, *serrana*, and *murciana*) of local Spanish stock, were members of five different herds which had suffered from epizootic outbreaks of the disease between 1990 and 1994 in Cáceres, Extremadura, Spain. After their isolation and identification, the strains were kept in the collection of the Department of Infectious Diseases of the Faculty of Veterinary Medicine at the University of Extremadura for further studies.

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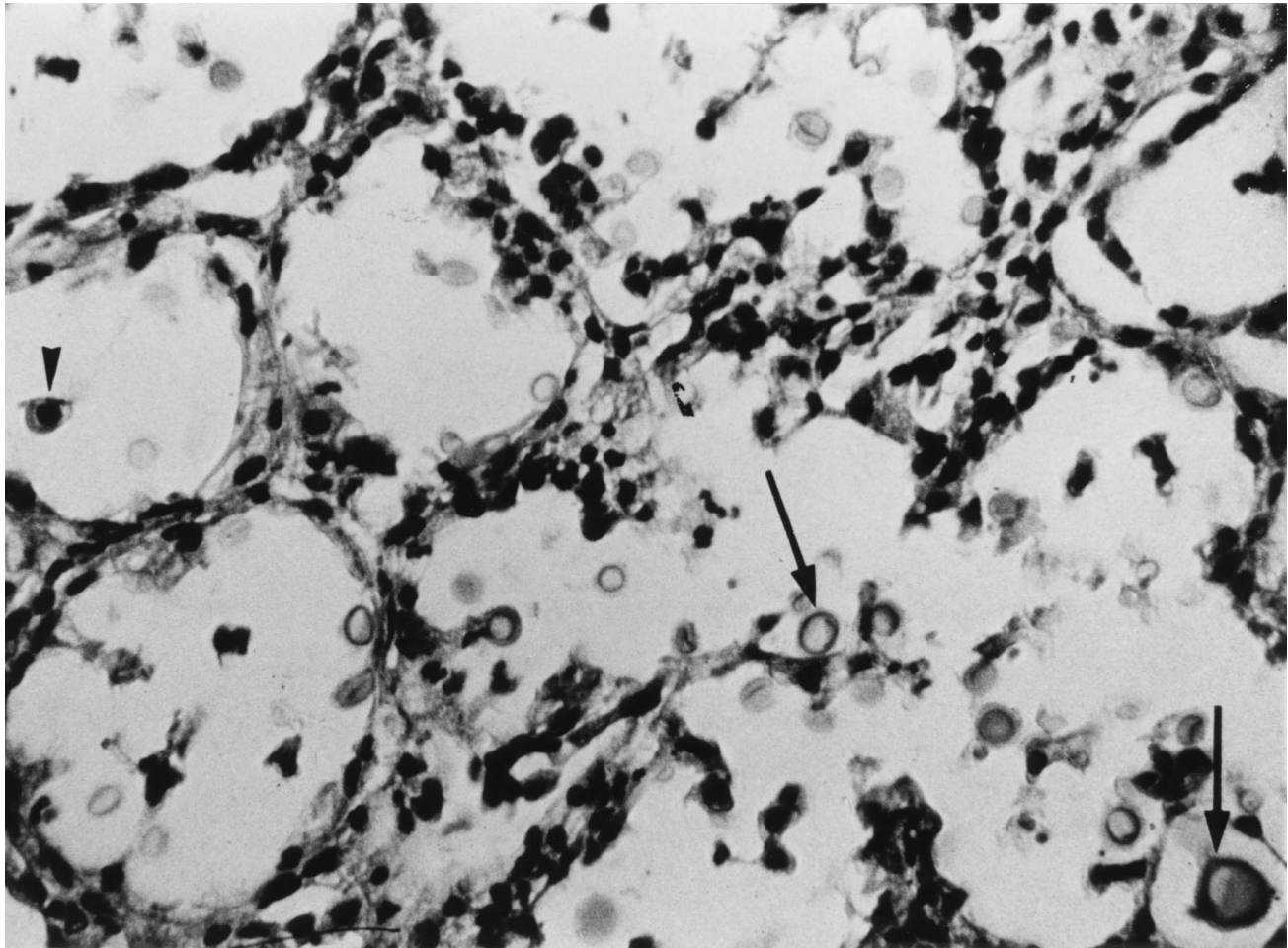


FIG. 1. Histopathology of lung tissue from an autopsied goat showing a high number of encapsulated yeast cells inside the alveoli. Hematoxylin-eosin stain. Original magnification,  $\times 400$ .

**Outbreaks.** Between 1990 and 1994, five outbreaks of severe pulmonary disease associated with cachexia were registered in which the members of five different herds of goats grazing in different geographical areas of the countryside in the province of Cáceres were affected. Although respiratory symptoms associated with cachexia were the predominant clinical picture in all outbreaks, liver and brain involvement was also documented in three of the five outbreaks. The infected animals showed congestive nasal mucosae with mucopurulent exudate, cough, dyspnea, anorexia, and severe weight loss causing cachexia and leading to death after a variable period of 2 to 6 weeks. Neurological symptoms included ataxia, mydriasis, blindness, and progressive paralysis. No mastitis was observed in the infected animals. The number of affected animals varied from 2.5 to 12% of the herd, depending on the outbreak. No specific antifungal treatment was given, although empiric antimicrobials (penicillin) were administered. All animals with clinical manifestations died, with a lethality rate of 100%. It is unknown whether there were animals with subclinical infections.

**Cryptococcal isolates.** One or two animals from each herd that had died spontaneously were selected for autopsy. Multiple bilateral lung infiltrates were found. In three animals, enlarged mediastinal lymph nodes were also observed, as well as edema in the brain and multiple whitish focal infiltrates in the hepatic parenchyma. Various samples were taken under sterile conditions for histopathological and microbiological studies. Direct microscopy of samples treated with 20% potassium hydroxide and Gram staining of the lung tissue revealed the presence of spherical yeast cells, some of which had buds. Staining with India ink showed a high number of capsulated yeast cells. Upon microscopic examination, lung (Fig. 1) and brain samples showed masses of yeast cells with no inflammatory or fibrotic margins. Pulmonary, hepatic, and cerebral tissue specimens were cultured in Sabouraud dextrose agar medium with chloramphenicol (Difco, Detroit, Mich.). After 48 h at 37°C, several mucoid whitish colonies were observed. India ink mounts revealed capsulated yeast cells.

**Identification procedures.** Thirteen isolates were identified as *C. neoformans* by the API 20 C Aux system (BioMérieux, Marcy L'Etoile, France). The strains

were maintained by periodic subculturing in Sabouraud agar glucose tubes until they were sent to the Mycology Laboratory of the Institut Municipal d'Investigació Mèdica (IMIM) in Barcelona, Spain, for typing. The strains were grown on niger seed (*Guizotia abyssinica*) agar medium and in Pal's medium with sunflower seeds (*Helianthus annuus*) (26). The production of a brown pigment due to the action of phenoloxidase was observed in both media. The positive urease tests, the auxanograms for sugars, and the sensitivity to cycloheximide were all characteristic of *C. neoformans* (Auxocolor; Sanofi, Pasteur, Paris, France). The biovariety study was performed by culture in CGB medium (L-canavanine-glycine-bromothymol blue) to determine the use of glycine as the unique source of carbon (16, 27) by the D-proline and the D-tryptophan assimilation tests as unique sources of nitrogen (7, 22). Serotyping was performed with the Crypto Check agglutination test (Iatron Labs Inc., Tokyo, Japan) (13). The serotyping results were confirmed by the Mycological Laboratory of the Institute of Tropical Medicine in Antwerp, Belgium. *C. neoformans* reference strains ATCC 90112, RV 56164 serotype A, RV 20185 serotype B, RV 45978 serotype C, and RV 68038 serotype D were used as quality controls.

## RESULTS

Data on the five outbreaks of predominantly severe pulmonary disease which occurred in different geographical areas of the province of Cáceres, Extremadura, are summarized in Table 1 and Figure 2. All infected animals presented severe pulmonary symptoms associated with cachexia as the most relevant clinical manifestations, although in three of the outbreaks, symptoms of central nervous system involvement were also present. Involvement of brain and liver tissues was con-

TABLE 1. Characteristics of the different outbreaks of predominantly severe pulmonary disease associated with cachexia<sup>a</sup>

Date of outbreak	No. of animals per herd	Clinical prevalence (%) <sup>b</sup>	Necropsy (no. of animals)	Localization in central nervous system	Isolation of <i>C. neoformans</i> <sup>c</sup>
1990	140	12	2	No	5
1991	250	2	1	Yes	2
1994	300	10	1	No	2
1994	200	?	1	Yes	2
1994	120	2.5	1	Yes	2

<sup>a</sup> The outbreaks in goats in different geographical areas of the province of Cáceres, Extremadura, Spain.

<sup>b</sup> Lethality rate, 100%.

<sup>c</sup> Number of strains from different tissues.

firmed by histopathologic examination and culture. With the use of Indian ink, it was possible to observe encapsulated yeast cells characteristic of *C. neoformans* in pulmonary and brain tissue specimens. Thirteen very mucinoid strains were isolated from the 10 lung tissue samples as well as from the brain and liver tissues of the six autopsied goats. The identification showed that the 13 strains were *C. neoformans*, since the patterns of assimilation of sugars, cycloheximide tolerance, production of urease, and growth at 37°C were characteristic of this species. Strains were stained brown in the *H. annus* and *G. abyssinica* media. All of them were positive by the CGB test, and they assimilated D-proline and D-tryptophan, indicating that they belonged to *C. neoformans* var. *gattii*. Agglutination tests with the Crypto Check system demonstrated that all of the strains were serotype B. These results were confirmed by the Tropical Medicine Institute of Antwerp, Belgium.

## DISCUSSION

Since Ellis and Pfeiffer (8, 9) isolated *C. neoformans* var. *gattii* from the environment in Australia, its natural habitat has been associated with *E. camaldulensis* (river red gum tree). This native species of eucalyptus is widespread in the south of Australia, and it has been exported and planted in areas of California, Mexico, and other parts of the world (24). The

isolation of *C. neoformans* var. *gattii* from nature has been reported from the tropical zones of central Africa and Brazil (18). However, it has also been found in temperate zones of countries, such as Uruguay (10), and has been shown to cause some cases of human cryptococcosis in Argentina (2). It has also been isolated from material obtained from another *Eucalyptus* tree, *E. tereticornis*, and from different substrata, including a wasp's nest (10).

In Europe, *C. neoformans* var. *gattii* has been isolated in Germany from a patient working with wood of imported tropical trees (14); from environmental samples in Apulia, a region of Italy (21); and from *Eucalyptus* sp. samples in Portugal (20). Up to the present time, there have been no reports of the isolation of *C. neoformans* var. *gattii* from the environment or autochthonous infections in humans or other animals in Spain, a country with a temperate climate. *Eucalyptus* trees coming from France had been introduced in Extremadura since the last century, but an extensive reforestation with *Eucalyptus* sp. took place between 1955 and 1977. *E. camaldulensis* is by far the predominant species, followed by *Eucalyptus globulus* (5). Since 1980, no more reforestation campaigns with *Eucalyptus* have been promoted. Whether *C. neoformans* var. *gattii* was brought into Spain by infected imported trees is unknown.

It should be noted that autochthonous strains of *C. neoformans* var. *gattii* were isolated from goats of local Spanish stock suffering from severe subacute or chronic respiratory symptoms associated in some cases with systemic disease. The various animals from which this variety has been isolated were all found to be grazing free in different zones of the countryside in a region located in the central western part of the Iberian peninsula. The five outbreaks occurred in different geographical zones from 1990 to 1994. This suggests that there is wide distribution of this pathogenic yeast in the geographical area to which reference has been made. Inhalation was the most likely mode of infection. The animals in one of the herds had been grazing in a eucalyptus grove, in which the predominant species was *E. camaldulensis*.

In all cases, serotype B was identified. The prevalence of serotype B over serotype C in *C. neoformans* var. *gattii* everywhere except in the south of California is well known (9, 15, 17). Exhaustive studies to isolate this variety from the environment have still not been conducted in Spain, but undoubtedly this will become a primary objective in the near future.

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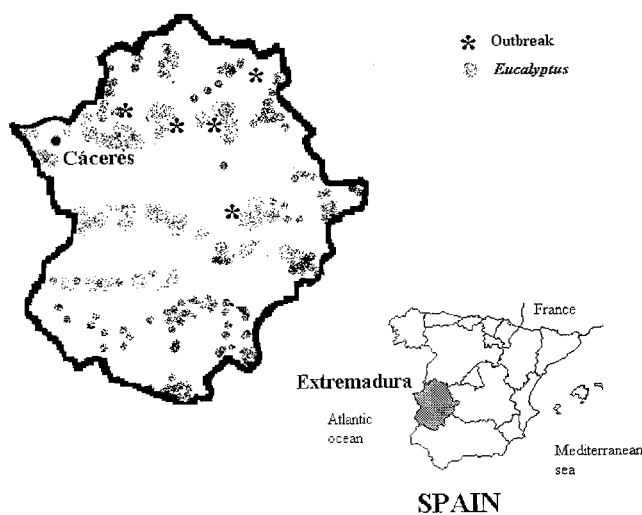


FIG. 2. Map of Extremadura, Spain, showing the locations of the five outbreaks of cryptococcal infections in goats and forested areas with *Eucalyptus* spp.

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