Serotyping of *Cryptococcus neoformans* Isolates from Clinical and Environmental Sources in Spain

TERESA BARÓ,* JOSEP M. TORRES-RODRÍGUEZ, YOLANDA MORERA, CONCEPCIÓN ALÍA, OLGA LÓPEZ, AND RAUL MÉNDEZ

Clinical and Experimental Mycology Research Group, Institut Municipal d’Investigació Médica, Autonomous University of Barcelona, Barcelona, Spain

Received 5 August 1998/Returned for modification 30 September 1998/Accepted 30 December 1998

We determined biovars and serotypes of 154 isolates of *Cryptococcus neoformans* from clinical and environmental sources from different areas of Spain. All clinical isolates belonged to *C. neoformans* var. *neoformans*. Serotypes showed an irregular distribution. *C. neoformans* var. *gattii* serotype B was isolated from necropsy specimens from goats with pulmonary disease.

*Cryptococcus neoformans* is an encapsulated yeast that is responsible for life-threatening infections, particularly in immunocompromised patients (7, 21). *C. neoformans* exists in two varieties, *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* (4, 32). These two varieties of *C. neoformans* (4, 32) are easily differentiated by their growth in L-canavanine-glycine-bromothymol blue agar (CGB) (19, 20) and agar with d-proline and d-tryptophan (9, 26). Based on the antigenic determinants of the polysaccharide capsule, serotypes A, D, and AD of *C. neoformans* var. *neoformans* and serotypes B and C of *C. neoformans* var. *gattii* have been identified (16). Differences between the two varieties with regard to pathogenicity and geographical distribution have been described. *C. neoformans* var. *neoformans* is responsible for most cases of cryptococcosis in immunocompromised patients, and *C. neoformans* var. *gattii* has been associated with infections in subjects with a normal immunologic status (10, 30). *Cryptococcus neoformans* var. *neoformans* has a worldwide distribution (3), whereas *C. neoformans* var. *gattii* has been reported to be restricted to tropical and subtropical areas (10, 11, 18, 29). Some autochthonous isolates have been obtained from temperate areas in Mexico and South America (2, 5, 14, 22) and Europe (1, 24). In Spain, the distribution of varieties and serotypes of *C. neoformans* isolates has not been previously assessed. We studied the distribution of biovars and serotypes of clinical and environmental isolates of *C. neoformans* from different geographical areas of Spain. Our findings were compared with the serotype distribution of *C. neoformans* isolates from several Latin American countries.

Rabbit polyclonal antibodies against each serotype, which were absorbed with the other serotypes, were prepared in our laboratory for serotyping the strains. A total of 154 *C. neoformans* strains isolated from different areas of Spain from 1988 through to 1997 were studied. Of these, 115 strains were from clinical human isolates; of these, only one strain was identified per patient, another 6 strains were isolated from goats with chronic pneumonia, and 33 isolates were obtained from environmental sources (soil and pigeon droppings). In 95% of the cases, samples were obtained from human immunodeficiency virus (HIV)-infected patients. All of them were intravenous drug users, except for one hemophiliac patient and one homosexual patient. The non-HIV-infected patients included recipients of organ transplantation as well as patients with hematological malignancies or solid tumors. A total of 110 strains from Central and South American countries were obtained from colleagues. They originated from Buenos Aires, Argentina (44 strains), Brazil (8 strains from Porto Alegre, 8 strains from Curitiba, and 28 strains from Rio de Janeiro), and Cuba (22 strains from Havana). All of the isolates were of clinical origin except one animal isolate from Cuba. *C. neoformans* serotype A strains ATCC 90112 and RV56164, serotype B strain RV 20185, serotype C strains RV45978 and NCPF 3170, and serotype D strain RV68038 were used as controls.

Strains were grown on niger seed (*Guizotia abyssinica*) agar medium and Pal’s medium with sunflower seeds (*Helianthus annuus*) (31). In both media, the production of brown-pigmented colonies due to the activity of phenoloxidase was observed. Positive urease tests, auxanograms for sugars, and sensitivity to cycloheximide (*Auxochrome; Sanofi, Pasteur, Paris, France*) were all characteristic of the *C. neoformans* isolates. The biovariety study was performed by culturing the isolates in CGB medium and noting their ability to use glycine as a unique source of carbon (19, 32). Disks impregnated with d-proline and d-tryptophan on carbon base agar were used for the study of these d-amino acids as unique sources of nitrogen (9, 26). The change of color of the CGB medium or growth of the strains in the presence of d-proline and d-tryptophan was assessed after 48 to 72 h of incubation at 25°C. Serotyping was performed by a slide agglutination procedure using polyclonal antibodies obtained from the immunization of male New Zealand White rabbits with heat-killed cells from the reference strains of serotypes A, B, C, and D. The method proposed by Evans (12, 13) with modifications was used for rabbit immunization. The rabbits were inoculated with daily doses of *C. neoformans* for 3 weeks, with progressively higher concentrations (10⁶, 10⁷, and 10⁸ CFU/ml) given each week, after jugular catheter insertion into the superior vena cava. Blood samples for determining the serum titers were obtained throughout the same catheter. One prophylactic dose of cloxacillin was given intramuscularly before surgery. Sera were absorbed with heterologous cells at 37°C for 2 h and left overnight at 4°C. After centrifugation, the supernatants were tested for the presence of antibodies by the slide agglutination test. The Crypto Check agglutination test (Iatron Labs Inc., Tokyo, Japan) (16, 17) was applied to 108 isolates for quality control of the procedure.

All isolates were urease positive and also gave a positive
reaction for phenoloxidase in the niger seed and Pal’s media. There was a complete agreement among the three methods used for determining the biovariety, and the biovars correlate with the serotypes. The strains of \textit{C. neoformans} var. \textit{gattii} grew, producing a color change in CGB medium, and all assimilated \textit{D}-proline and \textit{D}-tryptophan with enhanced growth around the disk. The serotype distribution of the Spanish isolates of \textit{C. neoformans} according to their source is shown in Table 1. Most of the human and environmental isolates belonged to serotype \textit{A}; only those isolated from goats were serotype \textit{B}. Complete agreement in results was found with the 108 isolates tested with the Crypto Check agglutination method. The geographical origin and serotype distribution of the clinical Spanish isolates are shown in Table 2. Distribution of clinical serotypes around the country was uneven (Fig. 1). Complete clinical data were obtained in 73\% of the Spanish cases. Only three of them were HIV negative, and all belonged to serotype \textit{A}. In the HIV-positive patients, serotype \textit{A} was predominant (59.3\%) but a high number of serotype \textit{D} (28.4\%) and serotype \textit{AD} (12.3\%) isolates were also found. No \textit{C} serotype isolates were observed. Serotypes of the clinical isolates of \textit{C. neoformans} from Brazil, Cuba, and Argentina are shown in Table 3. \textit{C. neoformans} var. \textit{gattii} was not found among the clinical isolates from Argentina and Cuba. One strain of \textit{C. neoformans} var. \textit{gattii}, however, was obtained from a necropsied cheetah (\textit{Acinonyx jubatus}) from the National Zoo of Havana. In contrast, 18 isolates of \textit{C. neoformans} var. \textit{gattii} were identified among the Brazilian strains, 14 of them having been isolated from HIV-negative patients. This was the first epidemiological survey performed in Spain on the prevalence and geographical distribution of the biovarieties and serotypes of \textit{C. neoformans}. As in other European countries in which epidemiologic studies of \textit{C. neoformans} have been carried out, \textit{C. neoformans} var. \textit{neoformans} is the cause of cryptococcosis in HIV-infected patients and other immunocompromised hosts. Until now, in Spain, no isolates of \textit{C. neoformans} var. \textit{gattii} have been observed in humans, but this variety has been isolated from goats with cryptococcosis suffering from severe pulmonary disease (1). This finding suggests that the autochthonous distribution of serotype \textit{B} of \textit{C. neoformans} is not limited to tropical and subtropical areas but also includes areas with temperate climates, such as Spain, Portugal, and Italy (23, 24). There seems to be a relationship between the distribution of five species of \textit{Eucalyptus}, including \textit{E. blakelyi}, \textit{E. camaldulensis}, \textit{E. gomphocephala}, \textit{E. rudis}, and \textit{E. tereticornis} (11, 27, 28) and \textit{C. neoformans} var. \textit{gattii} isolates. Most of these \textit{Eucalyptus} species are found in some Spanish geographical locations, but no isolates of \textit{C. neoformans} var. \textit{gattii} have been previously identified (6). In Europe, there have been a few reports in which autochthonous human isolates of \textit{C. neoformans} var. \textit{gattii} have been described (18, 25). The three methods used for the differentiation of the two varieties were accurate, and the results were in agreement. All isolates of the \textit{C. neoformans} var. \textit{gattii} assimilated \textit{D}-proline and \textit{D}-tryptophan and grew in CGB medium; therefore, any one of these methods could have been used. The assimilation test for \textit{D}-amino acids is easy to perform and less expensive than growth in CGB medium.

Table 1

<table>
<thead>
<tr>
<th>Source</th>
<th>Total no. of strains</th>
<th>No. (%) of strains of indicated serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humans</td>
<td>115</td>
<td>71 (62) AD 33 (29) 11 (9) 0 0</td>
</tr>
<tr>
<td>Animals</td>
<td>6</td>
<td>0 AD 0 0 AD 6 (100) 0 0</td>
</tr>
<tr>
<td>Environment</td>
<td>33</td>
<td>26 (79) 5 (15) 2 (16) 0 0</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Geographic region</th>
<th>Total no. of strains</th>
<th>No. (%) of strains of indicated serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barcelona</td>
<td>35</td>
<td>27 (77) 6 (17) 2 (6) 0 0</td>
</tr>
<tr>
<td>Valencia</td>
<td>11</td>
<td>0 0 0 AD 0 0</td>
</tr>
<tr>
<td>Mallorca</td>
<td>21</td>
<td>21 (100) 0 0 0 0</td>
</tr>
<tr>
<td>Sevilla</td>
<td>20</td>
<td>12 (60) 4 (20) 4 (20) 0 0</td>
</tr>
<tr>
<td>Madrid</td>
<td>17</td>
<td>6 (35) 9 (53) 2 (12) 0 0</td>
</tr>
<tr>
<td>Salamanca</td>
<td>1</td>
<td>0 0 1 (100) 0 0</td>
</tr>
<tr>
<td>Pais Vasco</td>
<td>9</td>
<td>4 (45) 2 (22) 3 (33) 0 0</td>
</tr>
<tr>
<td>Santander</td>
<td>1</td>
<td>1 (100) 0 0 0</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Country</th>
<th>Total no. of strains</th>
<th>No. (%) of strains of indicated serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>44</td>
<td>26 (59) 0 0 18 (41) 0</td>
</tr>
<tr>
<td>Cuba</td>
<td>22</td>
<td>21 (95) 0 0 19 (5) 0</td>
</tr>
<tr>
<td>Argentina</td>
<td>44</td>
<td>39 (88) 3 (7) 2 (5) 0 0</td>
</tr>
</tbody>
</table>

* Isolate of animal origin.
Spain). No serotype C isolates were found either in Spain or Europe. Data from American isolates also showed a higher prevalence of serotype A than serotype D in Brazil and Cuba. The exception to this pattern was for isolates from Argentina, possibly because Buenos Aires has a temperate climate similar to that of Spain. We would like to draw attention to the first isolation of *C. neoformans* var. *gattii* in a cheetah from the National Zoo in Havana (Cuba). This animal is another mammalian victim of cryptococcosis.

Currently, studies using molecular biology techniques are in progress to establish the genomic DNA patterns of *C. neoformans* and thus obtain a better understanding of the epidemiology of cryptococcosis in Spain.

This work was supported by grant 96/1991-01 from the Fondo de Investigaciones sanitarias (FIS) of the Spanish Ministry of Health.

We thank all of our colleagues who provided *C. neoformans* strains. We are grateful to Libero Ajello for constructive criticisms and critical review and to Marta Pulido for editing the manuscript and for editorial assistance.

REFERENCES


