V. CONCLUSIONES

Objetivo I

1. No ha sido fácil la recopilación de cepas de *Cryptococcus neoformans* ya que la mayoría de los laboratorios no las conservan. A pesar de todo se han obtenido 128 cepas de 115 pacientes, de 8 regiones geográficas de España. Varios de estos pacientes tenían más de un aislamiento en el tiempo. El período de los aislamientos abarca 10 años (1988-1997). El 95% de los aislamientos provienen de enfermos de sida.
2. La dificultad en la obtención de cepas de orígen animal ha sido mayor. Se han obtenido 13 cepas aisladas de 6 cabras con criptococosis de 5 rebaños de diferentes áreas de la provincia de Cáceres.

Objetivo II

3. El estudio ambiental de muestras de polvo doméstico de pacientes con criptococosis, enfermos VIH+ y controles sanos ha resultado negativo. Este substrato no representa un reservorio saprofítico de *C. neoformans* en nuestro medio ambiente.

Objetivo III

4. La aplicación del medio de L-canavanina-glicina-azul de bromotimol junto con la asimilación de aminoácidos ha permitido demostrar por primera vez que la variedad *gattii* de *C. neoformans* existe en nuestro país. Esta variedad ha resultado patógena para un número elevado de animales produciendo brotes epidémicos de criptococosis localizadas en Cáceres. Todas las cepas estudiadas pertenecían al serotipo B.
5. El hallazgo de *C. neoformans* var *gattii* autóctono da soporte a que esta variedad no está restringida a zonas tropicales y subtropicales.

Objetivo IV

6. Se han obtenido anticuerpos policlonales de buena calidad en conejos para los serotipos A, B, C, y D con un costo muy inferior a los comercializados.
7. La distribución de los serotipos no es homogénea en España. El serotipo A es predominante en muestras clínicas y ambientales, no obstante se ha encontrado una elevada tasa del serotipo D principalmente en Valencia y Madrid.
8. No se han hallado hasta ahora los serotipos B y C en muestras clínicas humanas. En los aislados de cabras todas las cepas fueron del serotipo B.

Objetivo V

9. El nivel global de resistencias *in vitro* de las cepas clínicas ha resultado muy bajo. No se ha encontrado ninguna cepa con CIM > 1 μg/ml, para anfotericina B, sin embargo las del serotipo A mostraron menor sensibilidad a este polieno.
10. La 5-fluorocitosina ha resultado el antifúngico menos activo sobre *Cryptococcus neoformans.*
11. El fluconazol ha mostrado menor actividad *in vitro* que el itraconazol. Tres cepas mostraron CIM>64 μg/ml. El serotipo D muestra menor sensibilidad a los dos triazoles que el resto de los serotipos. (P<0.00001)
12. Las cepas de *C. neoformans* var. *gattii* aisladas de cabras muestran una sensibilidad uniformemente baja para los 4 antifúngicos estudiados.
13. Las cepas ambientales mostraron sensibilidades similares a las clínicas.

Objetivo VI

14. El método colorimétrico *Sensititre* es fácil en su ejecución y en la lectura de los puntos de corte. Este sistema proporciona valores más bajos de CIMs que el método de referencia.

Objetivo VII

15. Solamente 5 cepas de *C. neoformans* de las 190 aisladas en España y LatinoAmérica mostraron CIM de 2 μg/ml para el nuevo antifúngico derivado del sordarín GM 237354. La variedad *gattii* mostró CIM, proporcionalmente más alta que los otros serotipos para el sordarín. Nuevamente no se hallaron valores ≥ 1 μg/ml. para la anfotericina B.

Objetivo VIII

16. La aplicación de la técnica de biología molecular del RAPD ha permitido demostrar que por lo menos existen 2 genotipos diferentes en la var. *gattii* aislada de cabras. Uno de los patrones parece ser el dominante ya que se ha hallado en todos los brotes epidémicos mientras que el otro patrón sólo se detectó en una zona.

17. La aplicación del RAPD en 7 cepas de 3 pacientes mostró su utilidad para discriminar entre diferentes aislamientos de un mismo paciente.

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Estudio de muestras de polvo doméstico para el aislamiento ambiental de *Cryptococcus neoformans*

Sr. Director,

La criptococosis es una infección fúngica que se adquiere habitualmente por vía inhalatoria. Esta infección se produce en la mayoría de los casos en personas inmunodeprimidas, en especial los pacientes con sida.

Cryptococcus neofonnans presenta dos variedades, C. *neofonnans* var. *neofonnans,* agente cau- sal de la criptococosis en humanos en nuestro país, cuyo nicho ecológico es con frecuencia las heces de aves sobre todo de paloma *(Columba livia)* [1] y C. *neofonnans* var. *gattii,* que se ha relacionado con la presencia de *Eucalyptus* de diferentes especies, *Eucalyptus blakelyi, Eucalyptus camaldulensis, Eucalyptus gomphocephala, Eucalyptus rudis* y *Eucalyptus tereti- cornis* [2-4]. Hasta ahora no se ha descrito ningún caso de criptococosis en humanos por esta última variedad en España aunque sí que se ha observado en animales [5].

En la búsqueda del reservorio de C. *neofonnans* var. *neofonnans* se han descrito aislamientos a partir del polvo doméstico en ciudades como Kinshasa (Congo)[6] y Río de Janeiro (Brasil)[7]. En ambas se ha comprobado una elevada tasa de aislamientos en los domicilios de pacientes con sida, considerándose como una potencial fuente de infección. Para conocer si esta levadura se encuentra en el ambiente doméstico de Barcelona, se ha realizado un estudio de 79 muestras de polvo casero.

Se recogieron las muestras manualmente en un recipiente esteril del dormitorio y sala de estar del domicilio. Se separaron las muestras en tres grupos: 62 obtenidas del domicilio de personas sanas, II de personas VIH positivas y 6 de pacientes afectos de sida con criptococosis en el momento de la recogida.

Las muestras se procesaron a partir de una suspensión de 0,05 g de polvo en 15 mI de solución salina fisiológica estéril con 0,3 gIl de cloranfenicol [7]. Se sembraron con un escobillón en dos placas de Petri, una que contenía un medio con extracto de semillas de *Guizotia abyssinica* y otra con el medio preparado con semillas de girasol *(Helianthus annuus)* [8]. Ambos medios permiten la observación de colonias pigmentadas de color marrón, debido a la actividad de la fenoloxidasa por parte de C. *neofonnans.* Las placas permanecieron a temperatura ambiente durante una semana y con observación diaria de las mismas.

Como control de calidad, se procesó, siguiendo la misma metodología, una muestra de polvo doméstico a la que se habían añadido lO ~ de una suspensión en solución salina deC. *neofor:- mans* a una concentración de 3 x IQ6 UFC/ml así como una muestra de polvo procedente de Río de Janeiro (Brasil) que contenía esta especie. Once muestras de polvo doméstico correspondien- . tes a dos pacientes con criptococosis y el resto de pacientes VIH + fueron remitidas ailaborato- rio de Micología de la Fundación Oswaldo Cruz (Río de Janeiro, Brasil), para su,procesamiento. De las 79 muestras de polvo analizadas 33 fueron positivas para levaduras. En ningún caso se aisló C. *neofonnans. CryptOCOCCltS laurentii* fue aislado en tres muestras y *Cryptococcus albi- dus* en una. Otras levaduras aisladas han sido *Candidafamata* (2 muestras), *Candida zeylanoi- des* (4 muestras), *Candida lipolytica* (1 muestra), *Candida* sp. (13 muestras), *Rhodotorula* sp. (8 muestras), *Aureobasidium* sp. (I muestra). Tampoco fue aislado C. *neofonnans* en las mues- tras procesadas en Brasil.

C. *neofonnans* var. *neofonnans* no ha sido aislado en el polvo doméstico de Barcelona, por lo que es de suponer que éste no constituye un reservorio de importancia para la transmisión de la criptococosis en ese medio. Posiblemente las condiciones bioclimáticas no favorecen el desarro- llo de esta especie, al revés de 10 que sucede en regiones tropicales. Para determinar las fuentes naturales de la infección en España es necesario que se continúen realizando estudios ecológicos y epidemiológicos.

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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 predom- inant 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 confir- mation 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 neofonnans is a capsulated yeast with a world- wide 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 *Filobasi- diella neofonnans* 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 immunodefi- ciency virus (4).

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

Cryptococcus neofonnans var. *neofonnans* 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 con- trast, C. *neofonnans* 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 *Euca/yptus camaldulensis* (9) and *Euca/yptus tereticomis* 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 differ- ent, 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 spo- radic 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 ofC. *neoformans* thatcauses 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 sys- temic disease occurred from 1990 to 1994.

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MATERIALS ANO METHOOS

Organisms. Thirtccn strains of C. */leofonna/1s* isolated from tissuc samplcs ()btaincd by autopsy ()f six goats with subacutc or chronic pneumonia associated with cachcxia from farms in dilJcrcnt districts were studied. These animals. of dilJercnt *Capra hircus* races *(verala, selTa/la,* and *murcia/la)* of local Spani~h st{}Ck. were mcmbcrs of five dilJcrent hcrds which had sulJercd from cpiz(){)tic ()utbrcaks ()f the disease bctween 1990 and 1994 in Cáceres. Extremadura. Spain. Aftcr their is()lati()n and idcntificati()n, thc strains were kept in the collecti()n ()f thc Dcpartmcnt ()f Infccti()us Discascs ()f thc Faculty ()f Vctcrinary Mcdicinc at thc Univcrsity ()f Extrcmadura f()r furthcr studics.

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Date of No. of Clinieal out break animal,:; prevalence (%,)"		pcr herd	Nccropsy *(m).* of animal,,)	u)Calization in ccntral nclVous systcm	(",liation of C. *11"0!(Jrnlall."*
1990	140	12	2	No	5
1991	250	2	I	Ycs	2
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1994	2() ()	?		Ycs	2
1994	120	2.5	1	Ycs	2

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firmed by histopathologic examination and culture. With the use of Indian ink, it was possible to obselVe 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, pro- duction 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.

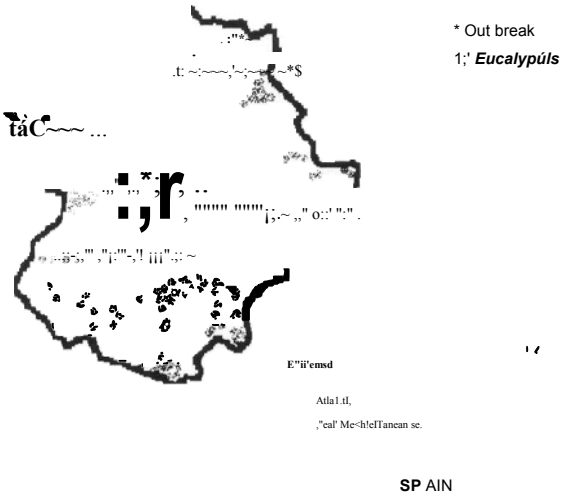
DISCUSSION

Since E1Iis and Pfeitfer (8, 9) isolated C. *neofonnans* 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 Califomia, Mexico, and other parts of the world (24). The isolation of C. *ne~forman.\'* var. *gattii* fr()m nature has been rep()rted fr()m the tr()pical zones ()f 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 ()f human cryptococcosis in Argentina (2). **It** has also been isolated from material obtaincd from an()ther *Eucaryptus* trec, *E. tereticomi.\"* and from different substrata, includ- ing a wasp's nest (10).

In Europe, C. *neoforman.\'* var. *gattii* has becn isolated in Gcrmany from a patient working with wood of imported trop- ical 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 authochthonous 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 *Eu- calyptus* sp. took place between 1955 and 1977. *E. camaldulen- sis* is by far the predominant species, followed by *Eucalyptus globulus* (5). Since 1980, no more reforestation campaigns with *Eucalyptus* have been promoted. Whether C. *neofomlans* var. *gattii* was brought into Spain by infected imported trees is unknown.

It should be noted that autochthonous strains of C. *neofor- mans* var. *gattii* were isolated from goats of local Spanish stock suffering from severe subacute or chronic respiratory symp- toms associated in some cases with systemic disease. The var- ious animals from which this variety has been isolated were al1 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 geograph- ical 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 spe- cies was *E. camaldulensis.*

In al1 cases, serotype B was identified. The prevalence of serotype B over serotype C in C. *neofomlans* var. *gattii* every- where except in the south of California is wel1 known (9, 15, 17). Exhaustive studies to isolate this variety from the environ- ment have stil1 not been conducted in Spain, but undoubtedly this wil1 become a primary objective in the near future.



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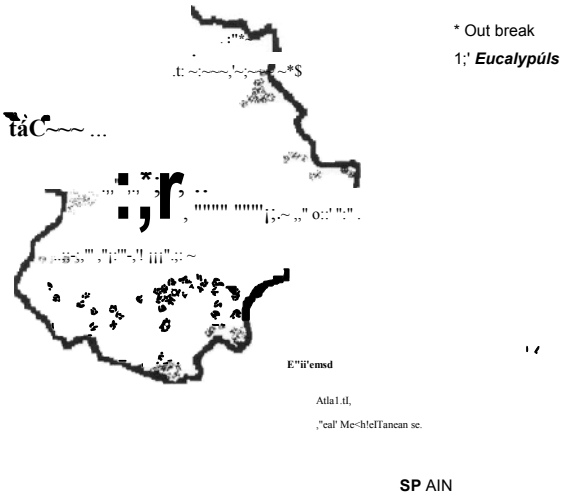

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First Identification of Autochthonous *Cryptococcus neoformans* var. *gattii* Isolated from Goats with Predominantly Severe Pulmonary Disease in Spain

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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 predom- inant 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 confir- mation 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 neofonnans is a capsulated yeast with a world- wide 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 *Filobasi- diella neofonnans* 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 immunodefi- ciency virus (4).

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

Cryptococcus neofonnans var. *neofonnans* 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 con- trast, C. *neofonnans* 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 *Euca/yptus camaldulensis* (9) and *Euca/yptus tereticomis* 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 differ- ent, 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 spo- radic 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 ofC. *neoformans* thatcauses 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 sys- temic disease occurred from 1990 to 1994.

MATERIALS ANO METHOOS

Organisms. Thirtccn strains of C. */leofonna/1s* isolated from tissuc samplcs ()btaincd by autopsy ()f six goats with subacutc or chronic pneumonia associated with cachcxia from farms in dilJcrcnt districts were studied. These animals. of dilJercnt *Capra hircus* races *(verala, selTa/la,* and *murcia/la)* of local Spani~h st{}Ck. were mcmbcrs of five dilJcrent hcrds which had sulJercd from cpiz(){)tic ()utbrcaks ()f the disease bctween 1990 and 1994 in Cáceres. Extremadura. Spain. Aftcr their is()lati()n and idcntificati()n, thc strains were kept in the collecti()n ()f thc Dcpartmcnt ()f Infccti()us Discascs ()f thc Faculty ()f Vctcrinary Mcdicinc at thc Univcrsity ()f Extrcmadura f()r furthcr studics.

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TABLE 1. Oistribution of thc scrutypcs of 154 C. *neofonnans* isolatcs from Spain with rcspcct to their sourccs

S<,urce	Total no. of strains	No. *(0/c)* of strains of indicated serotype				
		A	D	AD	B	C
Humans	115		33 (29)		0	
Animals	6		O		6 (100)	O
Environment	33		5 (15)			

reaction for phenoloxidase in the niger seed and Pa1'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 C. *neofonnans* var. *gattii* grew, producing a color change in CGB medium, and all as- similated D-proline and D-tryptophan with enhanced growth around the disk. The serotype distribution of the Spanish iso- lates of C. *neofonnans* according to their source is shown in Table 1. Most of the human and environmental isolates be- longed to serotype A; only those isolated from goats were serotype B. Complete agreement in results was found with the 108 isolates tested with the Crypto Check agglutination meth- od. 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 A. In the HIV -positive patients, serotype A was predominant (59.3%) but a high number of serotype D (28.4%) and serotype AD (12.3%) isolates were also found. No C serotype isolates were observed. Serotypes of the clinical isolates of C. *neofonnans* from Brazil, Cuba, and Argentina are shown in Table 3. C. *neofonnans* var. *gattii* was not found among the clinical isolates from Argentina and Cuba. One strain of C. *neofonnans* var. *gattii,* however, was obtained from a necropsied cheetah *(Acinonyx jubatus)* from the National Zoo of Havana. In contrast, 18 isolates of C. *neofonnans* var. *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 biova- rieties and serotypes of C. *neofonnans* .As in other European countries in which epidemiologic studies of C. *neofonnans* have been carried out, C. *neofonnans* var. *neofonnans* is the cause of cryptococcosis in HIV -infected patients and other immuno- compromised hosts. Until now, in Spain, no isolates of C. *neo- fonnans* var. *gattii* have been observed in humans, but this variety has been isolated from goats with cryptococcosis suf- fering from severe pulmonary disease (1). This finding suggests

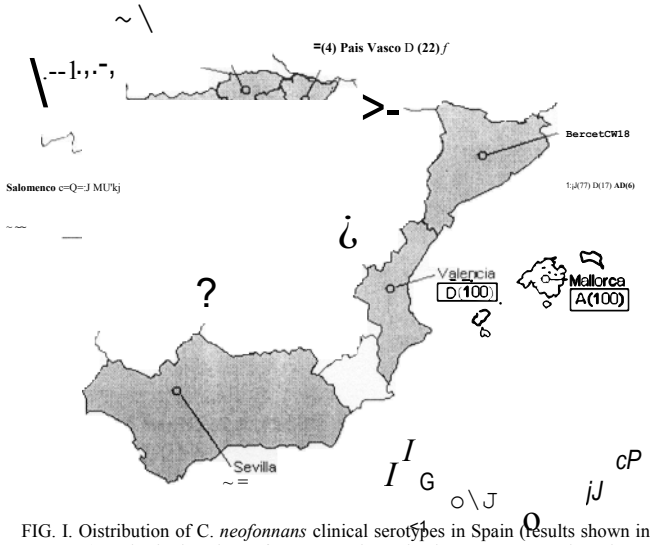

FIG. I. Oistribution of C. *neofonnans* clinical serotypes in Spain (results shown in percentages). Only single cultures from Santander and Salamanca were evaluated.

that the autochthonous distribution of serotype B of C. *neo- formans* is not limited to tropical and subtropical areas but also includes areas with temperate climates, such as Spain, Portu- gal, and Italy (23, 24). There seems to be a relationship be- tween the distribution of five species of *Eucalyptus,* including *E. blakelyi, E. camaldulensis, E. gomphocephala, E. rndis,* and *E. tereticomis* (11,27,28) and C. *neoformans var.gattii* isolates. Most of these *Eucalyptus* species are found in some Spanish geographical locations, but no isolates of C. *neoformans* var . *gattii* have been previously identified (6). **In** Europe, there have been a few reports in which autochthonous human isolates of C. *neoformans* var. *gattii* have been described (18, 25). The three methods used for the dilferentiation of the two varieties were accurate, and the results were in agreement. Ali isolates of the C. *neoformans* var. *gattii* assimilated D-proline and D- tryptophan and grew in CGB medium; therefore, any one of these methods could have been used. The assimilation test for D-amino acids is easy to perform and less expensive than growth in CGB medium.

Serotype A is prevalent in clinical specimens in Spain, but serotypes D (29%) and AD (9%) are frequent and predomi- nant in some areas (Fig. 1). This pattern also occurs with the environmental isolates, with serotypes D and AD frequently occurring. These data are consistent with the relative preva- lence of serotype D in some areas of France and Italy (8, 15, 33). We found an irregular distribution of serotype D among clinical isolates, with the highest prevalence of serotype D occurring in Valencia (eastern Spain) and Madrid (central

T **ABLE** 2. Geographical origin and serotype distribution of C. *neofonnans* among Spanish clinical isolates

Geographic region	Total no. of strains	No. (%) oí strains oí indicated serotype				
		A	D	AD	B	C
Barcelona	35	27 (77)	6(17)			
Valencia	11	O	11 (100)	O		
Mallorca	21	21 (100)	4 (20)	9		
Sevilla	20	12 (60)	(53)			
Madrid	17	6 (35)	1 (100)	2		
Salamanca	1	O	(22)			
País Vasco	9	4 (45)	O			
Santandcr	1	I (100)				

TABLE 3. Distribution of C. *neofonnans* serotypes in human and animal isolates from a West lndian and two South American countries

O>untry	Total no. of strains	No. (%) of strains of indicated serotype				
		A	D	AD	B	
Brazil	44	26 (59)			18 (41)	
Cuba	22	21 (95)			1"	
Argentina	44	39 (88)			(5)	O

u **Isolatc 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. *neofonnans* var. *gattii* in a cheetah from the National Zoo in Havana (Cuba). This animal is another mam- malian victim of cryptococcosis.

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

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Mots-clés : CI)Ptococcus ne~forman.~. Sérotypes. Sensibilité *in vitro.* Amphothéricine B. Flucytosine. Triazoles.

RELA TIONSHIP BETWEEN *IN VITRO* ACTIVITY OF FOUR ANTIFUNGAL DRUGS AND THE SEROTYPES OF *CRYPTOCOCCUS NEOFORMANS*

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SUMMARY ~

Objectives. The aim of this study was to assess the *in vitro* activity of four antifungal agents against 128 clinical strains of *Cryptococcus neoforman.v* isolated from different geographical regions of Spain. The susceptibility of strains has been related to the serotype found for each isolate.

Materials and methods. The MICs were determined by the broth microdilution technique by foll1owing the modified guidelines described in the National Committee for Clinical Standards (NCCLS) document M27-A, using **RPMI** medium + 2% glucose.

Results. There was no resistance to amphotericin B but serotype A was less susceptible to this antifungal agent (P < 0.05). Only 39% of isolates were ful1y susceptible to flucytosine, most of isolates being intermediate (60%). No statistical differences were found among serotypes.

The MIC in which 90% of the strains were inhibited by triazoles was 16 /!g/ml for f1uconazole and 0.25 /!g/ml for itraconazole. Twenty-two percent of the strains had MICs ~ 16 /!g/ml (2% MIC ~ 64 /!g/ml) for f1uconazole and 13% of the isolates had a MIC ~ 0.25 /!g/ml (0.9% MIC = 1 /!g/ml) for itraconazole. Serotype D was found to be less susceptible to both azoles (P < 0.00001).

Conc/usion. The present study showed that the serotype A isolates of C. *neoformans* were less susceptible to amphotericin B than the serotypes D and AD (P < 0.05). There was no statistical significance in the susceptibility to f1ucytosine for the different serotypes. Most of *Cryptococcus neofonnans* were not susceptible to this agent. For the triazole drugs. the serotype D is the least susceptible (P < 0.0000 1).

These results suggest a relationship between susceptibilities to some antifungal drugs and C. *neoformans* serotypes.

RÉSUMÉ : Relation entre I'activité *in vitro* de quatre antifongiques et le sérotype de divers isolats de *Cryptococcus neoformans*

Objectif. Le but de cette étude est de connaltre I'activité *in vitro* de l'itraconazole, Vingt-deux pour cent des souches ont une CMI ?: 16 quatre agents antifongiques sur 128 souches cliniques de *Cryptococcus* I!g/ml (2% CMI ?: 64 I!g/ml) pour le fluconazole et 13% ont une *neoformans* isolées de différentes régions géographiques de l'Espagne, CMI ?: 0,25 I!g/ml (0,9% CMI = I I!g/ml) pour I'itraconazole, Le La sensibilité de chaque souche est comparée avec le sérotype trouvé, sérotype D montre la plus basse sensibilité pour les azolés

Matériels et méthodes, Les CMI sont determinées par la technique (P < 0.00001),

m~!fiée de microdilution en bouillon du "Nat~~na~ .Comminee. :or *Conclusion.* Cene étude montre que les souches qui sont de sérotype Cllmlcal Standards (NCCLS) document M27-A uullsant le mllleu A de C. *neoformans* sont moins sensibles a I'amphotéricine B que les RPMI + 2% glucose. souches de sérotype D et AD (P < 0.05). II n'y a pas de corrélation

Résultats.II n'y a pas de résistance a I'amphotéricine B mais le statistique entre les différents sérotypes et la sensibilité a la flucytosine. sérotype A est moins sensible a cet antifongique (P < 0.05). Seulement La plupart des souches de C. *neoformans* ne sont pas sensibles a cet 39 % des isolats sont sensibles a flucytosine, la plupart des souches agent. Le sérotype D montre la plus faible sensibilité aux azolés (60 %) ont une sensibilité considérée comme interrnédiaire. On n'a pas (P < 0.00001).
trouvé de différence statistique entre les sérotypes pour cet agent Ces résultats suggerent une relation entre la sensibilité *in vitro* a anUfonglque. quelques antifongiques et les sérotypes de C. *neoformans.*

Par rapport aux triazoles, la CMI pour laquelle 90 % des souches sont
inhibées est de 16 I!g/ml pour le fluconazole et de 0.25 I!g/ml pour

INTRODUCTION

Cryptococcus neoformans is an opportunistic fungal patho- gen with a wide geographical distribution. This organism is the causative agent of cryptococcosis. Cryptococcal meningitis is the disease most frequently observed, but disseminated disease affecting any organ is also common, especially in human irnrnunodeficiency virus-infected patients (8, 21, 23). Prirnary treatrnent of acute disease with arnphotericin B, with or without flucytosine, is usually recornrnended, however it is associated with a high toxicity profile and failure in sorne patients (10,34). To prevent relapses, long-term follow-up prophylaxis rnainly with fluconazole (17) is used.

The developrnent of standard methods for routine suscepti- bility testing of c. *neoformans* is important to facilitate the choice of antifungal agents and to selecl lhe appropriate doses of these agents. particularly in the case of resistanl isolates (9, 32).

Few studies on the antifungal susceptibilities of clinical strains of C. *neoformans* have been performed in Spain (30) and in any case there is no information on the serotype distribution

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of isolates. The aim of the present work is to assess the *in vitro* activity of four antifungal agents, (amphotericin B, flucytosine, fluconazole and itraconazole) against 128 clinical strains of C. *neoformans* isolated from different geographical regions of Spain by using the reference microdilution method proposed by the National Committee for Clinical Laboratory Standards (NCCLS, M27-A document) (25). The susceptibility of each strain has been related to the serotype found for each isolate.

MATERIALS ANO METHOOS

Antifungal agents

Amphotericin B was provided by Squibb (Esplugues LL.. Spain). flucytosine by La-Roche (Hoffman-La Roche, Switzerland), fluconazo]e was from Pfizer Inc. (Central Research, Sandwich, U K.), and itracona- zole from Janssen (Research-Foundation, Belgium). Amphotericin B and itraconazole were prepared in 100% dimethyl sulfoxide (DMSO) and flucytosine and fluconazole in sterile water. Stock solutions were prepared as described in document M27-A (25). The antifungal agents were dissolved at a concentration 100 times that of the highest dose to be tested (1.6 mg/ml to amphotericin B and itraconazole; and 6.4 mg/ml for flucytosine and fluconazole) and stored at -70 °C. until thawing on the day of susceptibility testing.

Medium

RPMI 1640 medium (Sigma Chemical. St Louis. Missouri 63103 USA) with glutamine but not bicarbonate + 2% glucose buffered to pH 7 with 0.165 M/L N-morpholino propanesulphonic acid (MOPS) buffer (Sigma) was used in a11 studies.

Microtiter plates

Microbroth dilution testing was perforrned on sterile microdilution plates, (Nunc@J, Brand products, DK-4000 Roskilde-Denmark). Dilu- tions of each antifungal agent were made in RPMI 1640 medium and 100 *fll* dispensed into wel1s I to 10 of each row. The antifungal concentrations ranged from 0.03 to 16 flg/ml for amphotericin B and itraconazole, and 0.125 to 64 flg/ml for flucytosine and fluconazole, and were obtained by 10 two-fold serial dilutions. Drug-free medium was dispensed into wel1s II and 12. Wel1 II served as a sterility control and spectrophotometric blank and wel1 12 served as a growth control.

Patients and Organisms

One hundred and twenty-two clinical isolates of C. *neoformans* from different patients and six strains from three patients who had two different serotypes in successives episodes of cryptococcosis were tested to determine drug MICs. These isolates were obtained between 1988 and 1998 from different patients of II Spanish hospitals in Catalonia, the Basque Country, Valencia, Majorca, Andalusia and Madrid. In 95% of the cases, samples were obtained from human immunodeficiency virus (HIV) infected patients. AII of them were intravenous drug users except for one haemophiliac and one homo- sexual patient. The non-HIV-infected patients included recipients of organ transplantation as well as patients with haematological malignan- cies or solid tumours. The isolates had been previously identified according to standard procedures (5, 36). *Cryptococcus neoformans* (ATCC 90112), *Candida albicans* (ATCC 90028) and *Candida krusei* (ATCC 6258) were included as reference or quality control strains whenever a test was run.

Serotyping

The serotypes of c. *neoformans* were tested by a slide agglutination test with polyclonal sera prepared in our laboratory (6) and confirmed with a Crypto Check agglutination test (latron Labs Inc., Tokyo, Japan) (18, 20).

Inocula

All the isolates and control strains were grown on Sabouraud dextrose agar. (bioMérieux SA. 69280 Marcy I.Étoile-France) at 35 °C. for 48 h. The yeast inocula were adjusted to the 0.5 McFarland standard (1 x 10" -5 x 10" CFU/ml) confirming the cell density with the aid of a spectrophotometer. The yeast stock suspension was mixed for 15 seconds with a vortex mixer. and diluted I: 50 and further diluted I: 20 with RPMI 1640 medium to obtain the inoculum (I x 10J to 5 x 10J CFU/ml). This inoculum was diluted I: 1 when the wells were inoculated to obtain a concentration of 0.5 x 10J to 2.5 x 10J CFU/ml. An aliquot of 0.1 ml was added to each well of the microdilution tray.

Incubation times

The plates were incubated at 35 °C, and microdilution MICs were read after 48 h. for C. *albicans* and C. *krusei* and after 72 h. for c. *neoformans*.

Endpoint criteria

The readings of the endpoints were perforrned visually using a reading mirror and the absorbances were deterrnined spectrophotometri- cally (A420) after automatic agitation of the plates for 15 seconds with an automatic microplate reader (Labsystems, Multiskan MS, Finland). The MIC for amphotericin B was the lowest drug concentration in which there wasabsence of turbidity. The MIC of flucytosine and azoles was defined as the lowest drug concentration in a well that produced 80% reduction in turbidity compared with growth control (25, 27, '35).

As the breakpoint susceptibility values for C. *neoformans* have not yet been proposed by the NCCLS we adopted the breakpoint values proposed by the NCCLS document M27 A for *Candida* spp. to *Crypto- coccus* (9, 26,37) in order to have a defined group of values for analysing the results.

All the isolates presenting unusually high MICs were tested twice for confirrnation of results.

Statistical analysis

The chi-square test was used to compare proportions in contingency tables. Whenever an expected value was less than 5. Fisher.s exact test was used.

Kruskall-Wallis non-parametric ANOVA or the Mann-Whitney ..U" test were used to compare groups medians for continuous variables that did not fit a normal distribution.

RESULTS

The susceptibility of 128 clinical isolates of C. *neoformans* was tested with four antifungal drugs. *Table I* describe the MICs required to inhibit 50 and 90% of the isolates and the range of control strains for all antifungal drugs. MICs for the control strains agreed with the NCCLS proposed values (24).

The MIC distribution of amphotericin B, flucytosine, flu- conazole and itraconazole are presented *(fig. 1*). Two isolates of C. *neoformans* serotype A from Andalusia showed the highest MICs (ll!g/ml) to amphotericin B, the MIC90 was 0.5l!g/ml for this polyene. Flucytosine was less active against C. *neoformans* than amphotericin B and azoles ($P < 0.0001$). Only 39% of strains presented an MIC ~ 4 I!g/ml to flucytosine, 60% with an

TABLE I. -I/1 \.ilrO aclivilie.f (iffi)ur a/1lifu/1gal agenls agai/1sl 128 Cl)pIOCOCCU.f /leofOrman.f cli/1ical i.folale.f a/Id Ihree reference .flrain.f. Aclivilé in I.ilro de qualre anlifo/1giqlle.f .fUr 128 isolal.f clinique.f de CI)'PIOCOCCIlS ne(~f{!"nan.f el sur Irois .f{!IIChe.f de réfèrence.

Agenl and .flrain group		MIC (Jlg/ml)*		
		Range		90%
Amphotericin 8				
Clinical		0.03-1	0.125	0.5
C. *lIe~f(Jmlml.~*	ATCC 90112	0.25-0.5		
C. *albicall.~*	ATCC 90028	0.5-1		
C. *kru.,ei*	ATCC 6058	0.5-1		
Flucytosine Clinical				
C. *lIeoformalls*		0.25-32		
c. *albicans*	**ATCC 90112**	2.8		
C. *krusei*	**ATCC** 90028	0.5-1		
Fluconazole	**ATCC** 6058	8-16		
Clinical				
C. *neoformans*		0.5- > 64	8	16
c. *albicans*	ATCC 90112	2-8		
C. *krusei*	ATCC 90028	0.25-1 32-		
Itraconazole	ATCC 6058	64		
Clinical				
C. *neoformans*		0.03-1	0.06	0.25
c. *albicans*	ATCC 90112	0.03-0.06		
C. *krusei*	ATCC 90028	0.03		
	ATCC 6058	0.12-0.25		

* 50% *and* 90%. *MICs for which* 50 *and* 90% *of the strains. respectively, are inhibited.*

MIC of 8-16 ¡tg/ml, and one clinical isolate gave an MIC of 32 ¡tg/ml. This isolate was serotype D and came from Madrid. The study of susceptibility to the triazoles showed that fluconazole had MICs S 8 ¡tg/ml for 78% of the C. *neoformans* isolates tested (100 of 128), 16 to 32 *¡tg/ml* for 20% ofthese isolates (26 of 128), and ?; 64 ¡tg/ml for 2% (3 of 128). Two serotype A isolates and one serotype AD isolate had a MIC ?; 64 ¡tg/ml to fluconazole. Itraconazole had MICs of S 0.12 ¡tg/mI for 87% of the isolates (111 of 128),0.25 to 0.5 *¡tg/ml* for 12% (16 of 128) and only one serotype A isolate which was from Catalonia (Barcelona) had a MIC of 1 ¡tg/ml.

Of all geographical areas, the isolates from Valencia showed the highest susceptibility (73%; $p < 0.05$) to flucytosine. With respect to the azoles, none of these isolates showed a MIC S 0.125 ¡tg/ml to itraconazole and a MIC S 8 ¡tg/ml to fluconazole. Neither showed values of MIC ?; 1 ¡tg/mI and ?; 64 ¡tg/ml respectively ($P < 0.0001$).

The Kruska\l Wallis test was used for comparing the MICs of the antifungal agents with respect to the different serotypes. This analysis revealed that although a\l the isolates were sensitive to amphotericin B, serotype A tended to have a lower susceptibility (MIC median 0.25 *¡tg/ml)* than the other serotypes D and AD, (MIC median 0.125 ¡tg/ml) with $p < 0.05$. In spite of the fact that some investigators such as Nguyen and Yu (26) have suggested MIC values for C. *neoformans* equivalent to those of *Candida* spp. in order to considerer an isolate as susceptible, intermediate or resistant, in this study three break-points to antifungal drugs were defined, for flucytosine. MIC ~ 4 I.Lg/ml. 8-16 l.Lg/ml and ?; 32 l.Lg/ml. for fluconazole MIC ~ 8 l.Lg/ml. 16-32I.Lg/ml and ?; 64 I.Lg/ml. and for itracona- zole MIC ~ 0.125 l.Lg/ml. 0.25-0.5 l.Lg/ml and ?; 1 I.Lg/ml. These breakpoints were compared with the serotypes by using a chi-square test *(jig.* 2). There were no significant differences among the serotypes and susceptibilities for flucytosine. In contrast, serotype D was less sensitive to the azoles ($P < 0.00001$) as 60% of the strains were in the interval 16-32 l.Lg/ml to fluconazole and 0.25-0.5 I.Lg/ml to itraconazole.

DISCUSSION

The *in vitro* susceptibilities of C. *neoformans* to amphotericin B. flucytosine. fluconazole and itraconazole have already been described by different investigators (4. 7. 38). however. only very limited information is available for some countries such as Spain. As the ecology of C. *neoformans* and the epidemiology of cryptococcosis are different in many parts of the world it is important to know whether such differences also reflect varia- tions in susceptibilities to antifungal drugs.

One would expect the breakpoints selected for *Candida* not to be useful when applied to other organisms such as C. *neofonnans*. However. some papers use this criterion to evalu- ate the susceptibilities of C. *neoformans* (9. 26). **It** is generally accepted that *in vitro* values can influence the effectiveness of

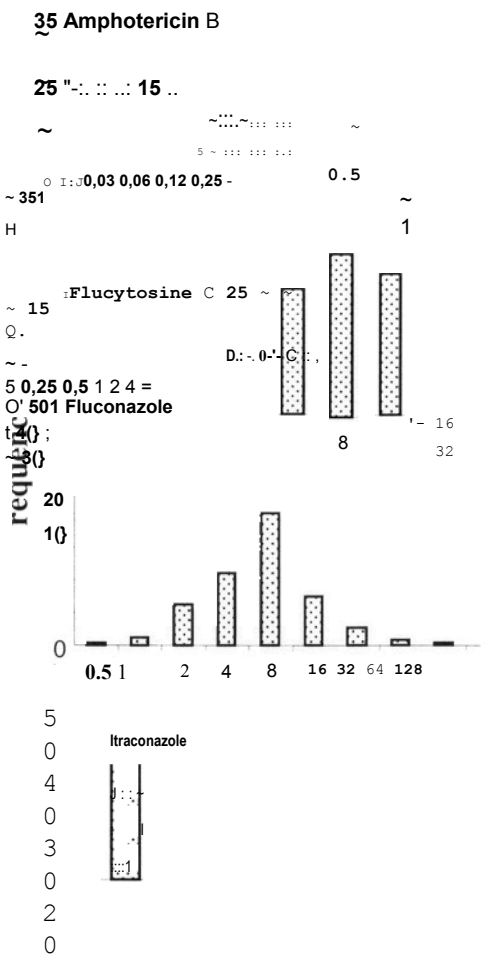

FIG. 1. -Distribution of MICs (l1g/ml) of amphotericin B. flucytosine, fluconazole and itrconazole for 128 clinical isolates of *Cryptococcus neoformans.*
Distribution des CMIs (l1g/ml) de l'amphotéricine B, de la flucytosine, du fluconazole et de \'itraconazole ou. 128 isolats cliniqués de *Cryptococcus neoformans.*

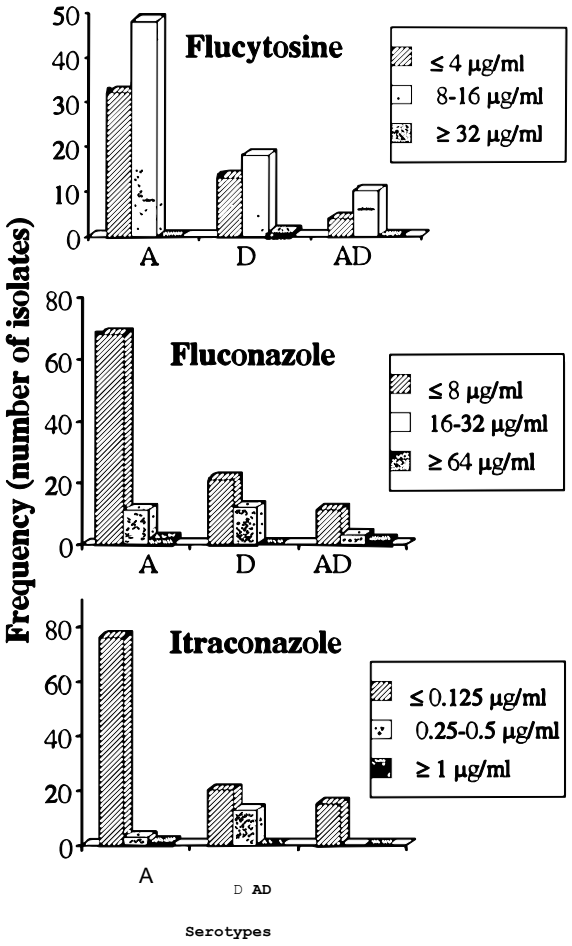

FIG. 2. -Susceptibility of the serotypes A, D and AD of *Cryptococcus neoformans* for flucytosine, fluconazole and itraconazole.
Sensibilité des sérotypes A, D et AD de *Cryptococcus neoformans* a la flucytosine, au fluconazole et a l'itraconazole.

antifungal therapy, susceptible strains do not always predict therapeutic success, but a high endpoint of C. *neoformans* will often predict therapeutic failure. (I, 13, 15, 19, 28.33, 39).

The relationships between serotypes of C. *neoformans* and antifungal susceptibilities are not well known. Poonwan *et al.* (31) studied the susceptibility of 50 selected strains of C. *neoformans* isolated from clinical specimens in Thailand and found only one resistant strain for flucytosine. but the authors did not indicate the serotype of this isolate.

The aim of the present study was to determine the MICs of the four most frequently used antifungal drugs for treating cryptococcosis. according to the different serotypes found in human clinical isolates of *Cryptococcus neoformans* in Spain. An additional objective was to relate the determined MICs to the uneven geographical distribution of cryptococci in this country (6). as also was described for Dromer *et al.* in France (II).

Cryptococcus neoformans serotype B isolates were not found in humans, although they have previously been isolated in Spain from animals (6).

Susceptibility could be determined by using different meth- ods but the standardised NCCLS M27-A method was chosen because comparisons with others studies can be made. and also because it offers the possibility of adapting the categories of susceptibilities for *Candida* spp to C. *neoformans* as other authors have published (9, 26). Obviously this categorisation of susceptibility must be confirmed by other studies including therapeutic responses (clinical aspects and treatment).

In agreement with other investigators (2, 14, 16) and based on our own experience, we found that RPMI 1640 medium with 2% glucose is a suitab1e medium for testing cryptococci. Although other authors suggest that Yeast Nitrogen Base (YNB) may enhance the growth of C. *neoformans.* in a pilot study we did not find any differences between RPMI and YNB (results not shown).

The results for the *in vitro* activity of amphotericin B showed that there were no Cryptococcus-resistant iso1ates to this poly- ene. Other authors have however found resistance to this drug

(9, 22, 32). Flucytosine gave the highest MICs compared with the other drugs (3), but only 0.8% of the isolates had MIC ;?: 32 ~g/ml and 60% of the strains were in the MIC interval 8-16 ~g/ml.

Resistance of C. *neoforman.*~ var. *ne()forman.*~ has begun to emerge in patients undergoing prolonged azole treatment (28). In our study only three isolates presented MICs ;?: 64 ~g/ml to fluconazole. If we compare these results with similar studies (29), it seems that the Spanish isolates were less sensitive to fluconazole (78%) than the African isolates (94%), and similar to the U.S. isolates (80%). The possibility of acquired resistance in patients on prolonged prophylactic fluconazole treatment should be considered. These results could be related to the extensive use of this azole in patients infected with human immunodeficiency virus (HIV) in Spain especially before the generalisation of the High Activity Antiretroviral Therapy (HAART). None of the patients included in this study ever received this kind of retroviral therapy.

In our study 95% of patients were HIV positive and we can assume that most of them had received antifungal treatment during the course of the disease, especially fluconazole for oropharyngeal candidosis. Considering that the samples were obtained over a period of lO years from II hospitals from various geographical regions of Spain, there is no unified antifungal therapy regime, nor primary prophylaxis, rejected by the majority of Spanish clinics.

Itraconazole showed higher *in vitro* activity in our study than fluconazole, only one isolate was found with a MIC = I ~g/ml to this triazole. This strain was found to have a MIC of 32 ~g/ml to fluconazole. Itraconazole is rarely administered for the treatment of cryptococcosis in Spain.

Regarding the serotypes, there were no resistant isolates to amphotericin B using the present methodology, but the statisti- cal analysis showed that the serotype A isolates of c. *neofor- mans* were less susceptible to this polyene than the serotype D and AD isolates ($P < 0.05$). So far this finding does not seem to be clinically relevant but could reflect a tendency in the follow up.

There was no statistical significance in the susceptibility to flucytosine for the different serotypes, and the only resistant isolate to this drug was from the serotype D.

Dromer *et al.* (12) suggest that individual and environmental factors may be associated with the serotype of c. *neoformans*. We found that, for the azole drugs, serotype D is the least susceptible ($P < 0.00001$). As the conditions of patients were similar (HIV + and the frequency of use of fluconazole for oropharyngeal candidosis) the MIC differences for azoles should be attributed to serotypes. These results however should be confirmed by a higher number of isolates.

The present results suggest that it is relevant to know the status of the susceptibility and resistance of c. *neoformans* to antifungal drugs in different parts of the world because signifi- cant differences could exist due to ecological and local factors as well as the antibiotic usage policy. Surveillance is also necessary for detecting the emerging resistance of C. *neofor- man.*~ strains to present or new antifungal drugs.

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In vitro susceptibility oí *Cryptococcus neoformans* isolates to five antiíungal drugs using a colorimetric system and the reíerence microbroth method

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Minimum inhibitory concentrations (MICs) of amphotericin B, 5-flucytosine, fluconazole, itra- conazole and ketoconazole were determined against 42 clinical isolates of *Cryptococcus neoformans* var. *neoformans* using the Alamar YeastOne colorimetric method and the NCCLS reference microdilution method. No strains with resistance to amphotericin B, itraconazole or ketoconazole were detected with either method. Using the reference method, the MICs of fluconazole were ~ 64 mg/L, whereas using the colorimetric method all MICs were ~ 16 mg/L. The MIC values of 5-flucytosine were also higher using the reference method (8-16 mg/L for 32% of isolates) compared with the colorimetric method. The percentage of agreement between the methods, using a difference of two dilutions, was 70.7% for itraconazole; 73.2% for ampho- tericin B, 80% for fluconazole, 88% for 5-flucytosine and 95% for ketoconazole. Overall, we con- clude that for fluconazole and 5-flucytosine, in a low but not insignificant number of isolates, results with the two methods are discordant, some isolates being found sensitive with the colorimetric test, but resistant with the reference method.

Introduction

Strains of *Cryptococcus neoformans* resistant to antifungal agents including flucytosine,I.2 fluconazole3.4 and ampho- tericin B5.6 have been shown to account for failure or relapse during azole treatment of cryptococcosis. This makes antifungal susceptibility testing even more import- ant in selecting and monitoring antifungal chemotherapy. Although standardized methods for broth macrodilution and microdilution testing of yeasts have been developed by the National Committee for Clinical Lab2ratory Standards (NCCLS),7 the availability of commercially prepared microdilution antifungal panels has been shown to enhance the ability to detect resistant isolates.8 Recently. a dried microdilution panel that incorporates an oxidation- reduction indicator (Alamar Bioscience Inc., Sacramento, CA, USA) has become available. Alamar YeastOne (Sensititre-Alamar YeastOne, Westlake, OH, USA) is a ready-to-use product; the 96-well microtitre plate has a colorimetric agent incorporated with the dried drugs. This produces a change from blue to pink in response to chemical reduction in the growth medium caused by the growing organisms in the inoculated wells. The first blue well in a row corresponds to the MIC of that drug. The antifungals included in the kit are amphotericin B, 5-flucytosine, fluconazole, itraconazole and ketoconazole. The Alamar colorimetric technique has demonstrated excellent agree- ment with the reference method when testing Gram-nega- tive bacteria9 as well as for the susceptibility testing of yeastslO and a high degree of intra- and interlaboratory reproducibility .10.11

We evaluated the reliability of the results obtained by the Alamar colorimetric method compared with the results generated by the reference microdilution method for 42 clinical isolates of C. *neoformans.*

Materials and methods

lsolates

A total of 42 clinical isolates of Co *neoformano\"* varo *neo- formans* (serotypes A, D and AD) obtained from AIDS

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palicnls during Ihe inilial diagnosis of cryplococcal infec- lion were tested. Quality control was ensured by testing *Ca1/dida albica1/s* A TCC 90028, *Candida kru.\"ei* A TCC 6258. *Candida parapsilosis* A TCC 22019 and *Candida 1/e(/- .r(/rman.\"* A TCC 90112 strains. Before Icsting, all isolales were subcultured on to Sabouraud dextrose agar (bio- Mérieux, Marcy l'Etoile, France) to ensure optimal growth characleristics. Slock suspensions were prepared in sterile phosphale-buffered saline (PBS) and adjusted to give a final inoculum concentration of 1 X 10(,-5 X 10(, cells/mL.

Colorimetric method

The Alamar colorimetric method was performed according to the manufacturer's instructions. Working suspensions were prepared by adding 20 fLL of stock yeast suspension to 11 mL of RPMI 1640 broth (American Biorganics, Niagara Falls, NY, USA) buffered to pH 7.0 with 0.165 M morpho- linepropanesulphonic acid (MOPS). This resulted in a final inoculum of 1.5 X 10-1-8 X 1ü3 cells/mL. The final concentra- tions of the antifungal agents were 0.04-4 mg/L of amphotericin B, 0.12-256 mg/L of fluconazole, 0.04-64 mg/L of 5-flucytosine and 0.008-16 mg/L of itraconazole and keto- conazole. The wells were reconstituted by the addition of 100 fLL of the inoculum suspension. After incubation at 35°C for 48 h for *Candida* spp. and 72 h for C. *neoformans,* MICs were determined by observing the lowest antifungal concentration preventing the development of a red colour (first blue well).

Reference microbroth method

The microdilution method was performed according to the recommendations of M27-A 7 using RPMI 1640 medium and 2% glucose in MOPS buffered to pH 7. Dimethyl sulphoxide (DMSO) was used as solvent for the anti- fungals, the stock solutions were prepared at 100 X the highest concentration to be tested. The final concentration was prepared from the antifungal stock solution in RPMI plus 2% glucose. The antifungal agents were dispensed in sterile, individually wrapped polystyrene round bottom assay plates. The stock solutions of antifungal agents were dispersed in the assay medium to obtain appropriate con- centrations in we"s 1-10 in each row; drug-free medium was dispensed in we"s 11 and 12. Well12 served as sterility control and we" 11 as growth control. The antifungal con- centrations were 0.03-16 mg/L of amphotericin B (Squibb, Princeton, NJ, USA), ketoconazole and itraconazole (Janssen Biotech, Beerse, Belgium), 0.125-64 mg/L of fluconazole (Pfizer Inc, New York, USA) and 5-flucytosine (La-Roche Laboratory Inc., Nutley, NJ, USA). The yeast inoculum was adjusted to 0.5 McFarland standard. A work- ing suspension was made at 1:100 dilution followed by a 1:20 dilution of a stock suspension with RPMI 1640 plus 2% glucose.

Thc il1oculalcd plalcs were incubaled for 72 h al *35°1* and readings werc lakcn daily. Absorbance was delel mined spcclropholomclrically al 420 nm afler agilalion (lhe plalcs. The MIC cndpoinl was dcfincd as lhe lowc~ drug concenlralion exhibiling approximalely *80°1.,* ((more) reduclion of growlh compared wilh lhe conlrol wcl For ampholericin B lhe MIC was defined as lhc IOWC1 concenlralion giving lOO(Yo inhibiliol1 (oplically clear).

Analysis of reSlllts

Discrepancies between MIC endpoints of no more tha two dilutions were used to calculate the percentage agree ment between the Alamar colorimetric method and th NCCLS reference microdilution method. The Student' *1* test for paired data was used for statistical analysis. Statis tical significance was set at $p < 0.05$. The SPSS compute program was used for analysis of data.

Results

After 48 h incubation, MIC for *Candida* spp. were withil the expected ranges in both the reference microdilutiol method and the Alamar colorimetric method. By the Ala mar colorimetric method, 400¡0 of C. *neoformans* isolate failed to produce any colour change in the growth contro well after 48 h incubation, so readings were performe(after 72 h incubation. Because the MIC scale is two dilu tions (0.016 and 0.008 ILg/L) lower than that of th4 reference method, values ~0.03 mg/L were considere(equivalent. Table I summarizes MIC ranges of the five anti fungals tested against 42 C. *neoformans* isolates deter mined by the reference microdilution method and th4 Alamar colorimetric method. The results are reported a MIC ranges, MIC5o and MI~, respectively. The highes MIC values were found for fluconazole and the lowest fo ketoconazole. The levels of agreement between the tW(methods for C. *neoformans* isolates are given in Table II Maximum and minimum agreements were found for keto conazole (95.10¡0) and itraconazole (70.70¡0), respectively.

There were statistically significant differences betweel the methods in both the range and distribution of MICs fo amphotericin B and 5-flucytosine, with a wider range aru significantly higher mean MIC values for the microdilutiol reference method as compared with the Alamar colori metric technique (amphotericin B, 0.143 versus 0.026 mg/l $p < 0.005$; 5-flucytosine, 6.316 versus 2.06 mg/L, $p < 0.0005$) In respect of the three azole compunds, lower mean MI(values *(*$P = 0.08$) were obtained for fluconazole with thl Alamar colorimetric method than with the referencl method, in which MIC values ~64 mg/L were obtained il 2.3 0¡0 of strains. Differences between the three azole agent were greater for MIC90 than MIC5o. All compound showed identical MIC5o values.

Table I. MIC distribution in 42 isolates oí *Cryptococcus neoformans* against five antiíungal drugs with the micro
Alamar-Blue colorimetric test (data in percentages)

MIC (mg/L)	amphotericin B Alamar NCCLS		fiuconazole Alamar NCCLS		Antifungals --- itraconazole - Alamar NCCLS		Alamar
0.008							7.1
0.016	38				2.3		14.
0.03	57.1	31.11			9.5	57.14	2
0.06	4.7	28.89	2.3		4	11.90	33.
0.125		13.33	2.3		5	14.29	3
0.25		22.22			3	9.52	38
0.5		4.44	2.3	4.26	3	7.14	4.7
1			7.1	17.02	7.1		2.3
2			14.2	10.64	2.3		
4			26.1	25.53			
8			38	23.			
16			4.7	4			
32				10.64			
~64				6.38			
Range Mean	0.016-0.06	<0.03-0.5	0.06-16	0.026 0.143 7.431 2.13	0.016–0.5	<0.03–0.5	0.08-0.2
MIC MIC50	0.03 0.06 4			0.5-64	0.101	0.10	0.047
MI~	0.03 0.25 8			8.44	0.06	0.06	0.03
				4	0.25	0.125	0.06
				1			
				6			

Tablc II. Agrccmcnt and diffcrcnccs ('X,) hclwccn thc colorimclric ¡Ino rcfcrcl1cc micr()oilulion mclhoos for flvc anl fungal orugs: amphotericin B. f1uconazolc. ilraconazole. kcloconazolc ano 5-f1ucytosinc against (', *11('(¡f(¡nllllll.\'* isolall

Oilutions		Amphotcricin B	Fluconazolc	Itraconazolc:	Kc!ocol1azolc	
3	agrccment	92.6.'{3	92.5	**H5.366**	I()()	95. 424
	differcnce	7.3171	7.5	**14.634**	()	HH. 762
2	agreement	73.171	**80**	**70.732**	**95.12**	II. 57I
	differcncc	26..'{29	20	**29.26H**	**4.~7~**	42. 9
	agrccment	43.902	72.5	**43.902**	**75.61**	16. **14**
	difference	56.09.,{	27.5	**56.098**	**24.39**	83. **~6**
	agreement	17.073	22.5	**9.7561**	**24.39**	**67**
	difference	82.927	77.5	**90.244**	**75.61**	**33**

Discussion

There are only a few studies on the susceptibility of C. *neo- formans,* so it is interesting to have data available on isolates from different sources and geographical distribu- tions.12 To compare results, however, it is necessary to use a similar and standardized methodology. Although the reference methods provide objective and comparable results, the availability of automated plate-reading tech- nology simplifies the procedures including MIC endpoint determination and avoids many manipulations of the standardized reference methods.l.l In isolates of *Candida* spp., the recently introduced colorimetric micromethod has shown a good reproducibility and correlation with macrodilution and microdilution methods.11 In a study sim- ilar to ours, Pfaller & BarrylO compared the NCCLS refer- ence method and the Alamar colorimetric technique in 600 clinical yeast isolates. The collection, however, included only four C. *neoformans* isolates. These authors found a 100% agreement between both methods in MIC5o of amphotericin B, fluconazole and 5-flucytosine. It should be noted that all four C. *neoformans* isolates were susceptible to all antifungal agents with MIC ranges 0.25-1.0 mg/L.

Testing of the susceptibility of C. *neoformans* to differ- ent drugs has been extensively studied with a variety of culture media, although the number of published reports of clinical resistance to amphotericin B in C. *neoformans* is surprisingly low. Lozano-Chiu *et al.:have* shown that both substitution of RPMI 1640 for antibiotic medium 3 in the microdilution variant of the M27-A method and use of the Etest agar diffusion methodology permits detection of amphotericin B-resistant *Candida* isolates. Only antibiotic medium 3, however, permitted consistent detection of amphotericin B-resistant C. *neoformans.* Because RPMI 1640 is the medium used in the colorimetric method, this may be the reason for the low number of resistant isolates found in the present study. Although all isolates were susceptible t() amphotericin B, MIC rangcs were lower for thc Alamar test than for the reference microdilution method in which thc RPMI 1640 medium was also used.

Similar findings were obtained with 5-t1ucytosine, MI(varying between 0.06 and 4 mg/L for the Alamar test ar between 0.5 and 16 mg/L for the reference method (33% , isolates showed MIC values ~8 mg/L). These differenc, were cven greater for MICIj(J.

With amphotericin and itraconazole the distribution I MICs for both methods was largely different. It should t noted, however. that no discrepancies in resistance (sensitivity of c. *l1eoformans* strains to these two antifung agents were observed. Inlcontrast, MIC values of flucon; zole and 5-flucytosine obtained with the Alamar test and tt reference method were quite similar, but with the imporlal drawback that some strains apparently sensitive to flucon; zole and 5-flucytosine with the Alamar test were resistant I the agents when tested with the reference method.

If the MIC breakpoints for fluconazole resistance in (*albical1s* mucosal infections (~64 mg/L)14 are applied, t~ number of C; *neoformans* strains classified as resistant

low. Davey *et al.ls* have reported an incidence of 5.6%, s that it may be expected that the 2.3% found in the presel study with the reference method is more close to the actu: situation than the total absence of resistance observed wit the colorimetric micromethod.

The degree of agreement of the two methods in terms (absolute values is very low when concentrations are ident cal (9-24%), increasing to 24-56% when there is a diffe' ence of one dilution, to more than 70% for two dilutior and between 92.5-100% for three dilutions. Thereforl when different methods are used, identical MIC valu(should not be expected; what is necessary is that chang(in absolute values do not cause a change in scoring of a isolate as resistant or susceptible, and vice versa, as h~ occurred, especially with fluconazole.

In summary, the Alamar colorimetric method is a usefl tool in the study of MICs of the five systemic antifung¡ agents available for systemic treatment of fungal infection With c. *l1e()f()rmans* however, the distribution of MICs (fluconazole and 5-f1ucytosine does not provide sufficientl concordant results in a low but not negligible number (isolates.

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In vitro susceptibility of *Cryptococcus neoforlnans* serotypes to GM 237354 derivative of the sordarin class

Empfindlichkeit van *Cryptococcus neofomzans-Seratypen in vitro* für das Sardarin-Derivat *CM* 237354

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Key words. *Cryptococcus neoformans,* serotypes, antifungal susceptibility, sordarin, amphotericin B. **Schlüsselworter.** *Cryptococcus neofo17llans,* Serotypen, Antimycestische, Empfindlichkeit, Sordarin, Amphotericin

SUInInary .*In vitro* susceptibility to the sordarin derivative GM 237354 and amphotericin B were tested in a total of 190 *Cryptococcus neofo1mans* clinical isolates from different geographical areas of Spain and South Arnerican countries. Minimal inhibitory concentrations (MICs) were obtained using the NCCLS reference microbroth dilution method and analysed according the serotypes of *Cr. neofo1mans.* The MICs for amphotericin B were lower than 1.0 J.1g ml-1 (MIC9o% 0.5 J.1g ml-1, MIC5o% 0.125 J.1g rn1-1) but five isolates showed MICs of 2.0 J.1g rn1-1 to GM 237354 (MIC9o% 1.0 J.1g ml-1, MIC5o% 0.5 J.1g ml-1). *Cryptococcus neofo1mans* var. *gattii* serotype B, was significantly less susceptible than A and AD serotypes *(P*= 0.047 and *p*= 0.022, respectively).

ZusalnJnenfassung. An 190 klinischen Isola- ten von *Cryptococcus neofo7mans* aus unterschiedlichen Regionen Spaniens und Südamerikas wurde die Empfindlichkeit *in uitro* für das Sordanin-Derivat GM 237354 im Verg1eich zu Arnphotericin B getestet. Die MHK-Werte wurden mittels NCCLS- Mikroverdünnungs- Referenzmethode erhoben und ein Vergleich nach *Cr.* neofo7mans-Serotypen durch- gef\ihrt. Die MHKs f\ir Arnphotericin B 1agen unter 1.0 ~g ml-1 (MHK9o% 0.5 ~g m1-1, MHK5o% 0.125 ~g m1), aber funf Iso1ate zeigten MHKs von 2.0 ~? m1-1 fur GM 237354 (MHK9o% 1.0 ~g m1- , MHK5o% 0.5 ~g ml-1). *Cryptococcus neofonnans* var. *gatti* Serotyp. B war signifikant weniger empfind1ich a1s die Serotypen A und AD *(P*= 0.047 bzw. *p*= 0.022).

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lntroduction

Sordarin derivatives are a new class of antifungal drugs that appeared to be selective and potent inhibitors of the fungal protein synthesis system [1,2]. Compound GM 237354 have been isola- ted and characterised from a broth obtained from the culture of the mould *Graphium putredinis.* The broth media inhibited fungal protein synthesis of *Candida albicans* and the following isolation of the active component showed some similarity to sordarin and zofimarin, but the novel antibiotic was designed with the number 237354. **In** first studies GM 237354 have shown *in vitro* activity against C. *albicans, Candida tropicalis, and Cryptococcus neofo1mans* with minimal inhibitory concentrations (MICs) ranging between 0.008 and 1.0 ~g ml-l. **In** an experimental model of mice with systemic candidosis a therapeutic effect was achieved following subcutaneous injections of 40 mg kg-1 [3].

As sordarins seem to be promising antifungal drugs, the objective of this study was to analyse a large number of *Cr. neofo7mans* isolates for their MICs against sordarin derivatives comparing it with amphotericin B and considering the source of yeasts and serotypes of the strains.

Materials and methods

Isolates

A total of 190 clinical isolates of *Cr. neofonnans* were included in the study. Clinical samples were mostly recovered from HIV -infected patients with crypto- coccosis from different countries including Spain *(n* = lI7), Argentina *(n* = 29), Brazil *(n* = 22), and Cuba *(n* = 22). Of these, l75 were from the *neofonnans* variety (serotype A 72%, serotype D 11.5%, serotype AD 8.4%) and the remaining l5 belonging to the *gattii* variety, serotype B. Method for serotyping have been previously described [4] .

Five reference isolates of *Candida albicans* (A TCC 90028), *Candida krusei* (A TCC 6258), *Candida para- psilosis* (A TCC 220 19) and *Cryptococcus neofonnans* (A TCC 90 l12 and R VB 20 l28) were included for quality control. They were used each time the test was done.

Methodology

For preparing the inoculum the yeast were grown on Sabouraud glucose agar plates for 24 h for *Candida* spp. and 48 h for *Cr. neofonnans,* Yeast concentrations were prepared for at least five colonies in sterile saline and adjusted at 0.5 McFarland standard. The inocula were adjusted to a concentration of 1-2.5 X 103, An aliquot of 0,1 ml was added to each well of the microdilution tray.

Amphotericin B in sterile powder was provided by Squibb Laboratories (Esplugues del Uobregat, Bar- celona, Spain); GM 237354 was obtained from GlaxoWellcome, S.A. (Tres Cantos, Madrid, Spain) as sodium salts that were initially solubilized in dimethyl sulfoxide (DMSO) at a concentration of 5 mg ml-1 and then diluted in medium to the appropriate concentration. Solutions were prepared just before use. The microdilution method was carried out and interpreted according to the recom-mendations of the National Committee for Clinical Laboratory Standards (M27-A document) [5].

RPMI 164o-g1utamine plus 2% glucose, in 3-[N-Morpholino] propanesulfonic acid (MOPS) buffer to pH 7 was used as culture medium. Antifungal stock solutions were prepared in water (GM 237354) or DMSO solvent for amphotericin B at concentrations 100 times higher than the working solution, The final concentration was prepared from the antifungal stock solution in RPMI and 2% glucose, with a range between 0.03 and 16 ~g ml-l. Antifungal agents were dispensed in sterile, wrapped polystyrene flat-bottom assay plates in wells I to 10 in each row; drug-free medium was dispensed in wells II and 12, the latter serving as a sterility control, and well II as growth control, The inoculated plates were incubated for 2+- 72 35 °C. Absorbance were determined wit automated plate reader at 414 nm for 237354, after agitation of the plates (Mult Labsystem MS, Finland). MIC was defined (lowest concentration in micrograms per millili the antifungal that inhibits development of 9~ growth in comparison with the growth C((when the antifungal prevents any visible gr(For amphotericin B the MIC was 100(inhibition, when the medium was optically
additional automated test was also carried (620 nm.

Analysir of results

The Student's *t-test* for paired data was use statistical analysis. Statistical significance w $p < 0.05$. The SPSS computer program (SPS~ Chicago, IL, USA) was used for analysis of d

Results

The MICs of amphotericin B for the control q strains agreed with the NCCLS defined v; Repetition of the tests did not give more thal dilution of difference for both antifungals. ranges of MICs to amphotericin B were eq¡; lower than 0.03 to 1 ~g ml-1, with a geon mean (SD) of 0.11 (0.17) ~g ml-1. The MI was 0.5 ~g ml-1 and MIC5o% was 0.125 ~g For the sordarin derivative GM 237354, the] was 0.03 to 2.0 ~g ml-1 , the highest MICs found in onli five isolates, with a mean of (0.37) ~g rnl- and MIC9o% and MIC500/o o and 0.5 ~g ml-1, respectively. The distributi, MICs to this product according the seroty¡ presented in Table 1.

There were no statistically significant differ between serotypes A and D *(P*= 0.9846), D AD *(P*= 0.3013), A and AD *(P*= 0.2274) or] D *(P*= 0.12). No differences were found acco the geographical origin (Spanish or Americ~ the isolates. Serotype B of *Cr. neoformans* var showed significantly higher MIC values serotype A *(P*= 0.0475) and serotype *(P*= 0.022).

Discussion

With the use of the standardized method susceptibility testing it is now possible to c reproducible and accurate results in ord. determine the levels of susceptibility of resis or *Cr. neofo7man.*\. [6, 7] .

Table 1. Distribution of MICs (μg ml^{-1}) of GM 237354, sordarin antifungal for 190 isolates of *Cryptococcus neoformans* according to serotypes

Serotypes	*n*	Range	Mean (SD)	$MIC_{90\%}$	$MIC_{50\%}$
A	137	0.003–2.0	0.537 (18.6)	1.0	0.5
B	15	0.5–2.0	0.833 (3.5)	1.0	1.0
D	22	0.06–2.0	0.587 (3.3)	1.0	0.5
AD	16	0.25–1.0	0.5 (4.1)	1.0	0.5

Over recent years, the lack of effective therapy for AIDS patients has prompted research into new antifungal molecules, as in the case of sordarin [2] . In this case the higher activity of this drug against *Pneumocystis carinii* and some dimorphic fungi, increased interest in the product [8, 9] .

The *in vitro* activity of GM237354 against *Cr. neofo7mans* has been investigated in only 20 strains for which the origin and biotype or serotype were not explained [8] .

Varieties and serotypes of *Cr. neofo7mans* are important with regard to ecology and epidemiology and virulence. Genetic analyses resulted in the new variety *grubii* being proposed for the present A serotype [9]. Up to now the correlation of *Cr. neofo7mans* serotypes with susceptibility or resist- ance to antifungals has merited little attention [10-13].

In our study, all the isolates showed MIC values for amphotericin B that were lower than 1.0 J.lg m1-1 with MIC9o% of 0.5 J.lg m1-1 confirm- ing the virtual absence of resistance of *Cr. neofo7mans* to this polyene. With GM 237354 in five isolates the MICs presented values of 2 J.lg m1-1, and the MIC9o% was 1.0 J.lg m1-1. These values were higher than the results observed in the previous study [8] in which the MIC range was 0.015-{).25 J.lg m1-1 and MIC9o% was 0.25 J.lg m1-1. These differences in susceptibility could be explained by the larger number of isolates tested in our study, and by the geographical diversity of strains and serotypes. Moreover, Herreros *et al.* [8] used yeast nitrogen base as the culture medium instead of RPMI 1640.

The clinical significance of the higher MICs observed needs to be determined in an animal model.

When the susceptibility was correlated to sero- types it was found that the MICs associated with the B serotype were higher in comparison with the A and AD serotypes. This tendency should be confirmed with a larger number of *Cr. neofo7mans* serotype B isolates.

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Cartas al Director

Caracterización molecular de *Cryptococcus neoformans* var. *gattli* causante de brotes epidémicos de criptococosis en cabras

Sr. Director,

Entre 1990 y 1994, en la provincia de Cáceres (España), se produjeron varios brotes de neumonía grave asociada a caquexia que afectó un número variable de cabras -entre el 2,5 y el 12% de las integrantes de los rebaños-. En algunos casos también existía una afectación encefálica y hepática y se registró una elevada tasa de mortalidad de los animales enfennos [I). La identificación de aislados obtenido"" por necropsia de animales, en cinco de los brotes, demostró que el agente etiológico era una levadura capsulada, identificada como *CryploCoCCUS neoformans.* Posterionnente el análisis bioquímico y serológico de 13 cepas ais- ladas de seis cabras, que se habían conservado en la colección de la Facultad de Veterinaria de la Universidad de Extremadura, pennitió comprobar que corres- pondían al serotipo B de la variedad *gallii* [2].

La demostración de que C. *neoformans* var. *galtii* existe en nuestro país, y que es capaz de ocasionar infecciones graves en animales sin otras patologías pre- vias, constituye un hallazgo destacable que enriquece la infonnación referida a la epidemiología de la criptococosis.

Para caracterizar las cepas aisladas se procedió a un análisis molecular utilizan- do la técnica del *Random Amplified Polymorphic DNA* (RAPO). Para ello se efectuó la extracción del AON de todos los aislamientos mediante la técnica de Lehmann *el al.* [3] modificada. Se efectuó una amplificación por PCR siguiendo la técnica de William *el al.* [4] con cuatro diferentes cebadores: CNI (5'TACCCCCGCCCATA1TCCAT3'.), 5S0R (5' ATGGGAA- TACGACGTGCTGTAG 3'), CI (5' ACGGTACACT 3') y C3 (5'GTlTCCGCCCC 3'), estos dos últimos se emplearon de fonna simultánea. Para la amplificación se utilizó un tennociclador (Perkin-Elmer Cetus, EE.UU.) realizándose 40 ciclos y el material obtenido se de analizó por electroforesis en gel de agarosa al 2% con tinción de bromuro de etidio. Después de una detallada observación visual, las imágenes se escanearon para ser procesadas en el sistema computarizado GEL DOC 1000, Molecular analysis (Bio-Rad Laboratories, Califomia USA).

Se detenninó el tamaño molecular de los fragmentos de ADN amplificados. La repetitividad de la técnica se confinnó por la amplificación del AON de un mismo organismo en tres ocasiones diferentes. Los fragmentos de dudosa inter- pretación no fueron asumidos como elementos de discriminación de los patrones [5,6).

Los datos proporcionados por el análisis de imagen han pennitido agrupar las cepas con idéntico perfil electroforético en dos diferentes patrones genotípicos que han sido denominados arbitrariamente A y B. Cuando se disponía de dos aislamientos de un mismo animal, el patrón genómi- co fue el mismo. El patrón A ha sido el dominante puesto que en todos los bro- tes se ha demostrado la presencia del mismo; sin embargo, en uno de los episodios ocurrido en la localidad de Vera, en que fue posible disponer de varias cepas aisladas de dos animales diferentes, se encontró el segundo patrón, o B (Tabla I).

Estos resultados sugieren que, con la metodología utilizada, se ha demostrado que en las diferentes zonas de Cáceres donde tuvieron lugar los brotes epidémi- cos' existe un tipo dominante *de* C. *neoformans* var. *gattii,* sin embargo esta cepa coexiste en la misma área por lo menos con otro tipo genómico.

Los resultados obtenidos, plantean diversas incógnitas como la de detenninar el nicho ecológico de esta especie en la naturaleza en Cáceres y en otras partes de la península ibérica y conocer la diversidad gen ética de las mismas, para poder relacionarla con la capacidad infectiva de esta especie.

T.bla 1 **Distribución** de kJs **aislamientos** de ***Cryptococcus n60fOlmans* var. *gattii* obtenido** de la **necrop- sia** de **cabras afectadas** de **criptococosis** y **el palrón gen6mico** de **las mismas detenTlinado utilizando el *Random Amplified Polymotphic ONA.***

Animar	Cepas **Muestra**	Localidad	Cebador utilizado SSOR CN1	C1+C3		Patrón
1	48A	Pulmón Pescueza Pulmón	III	I	VI	A
1	49A	Pescueza Pulmón Serradilla	III	I	VI	A
2	5OA	Pulmón Serradilla Hígado Casas	III	I	VI	A
2	6OA	de Millan Encéfalo Casas de	III	I	VI	A
3	51 A	Millan Pulmón Madroñera	III	I	VI	A
3	52A	Pulmón Madroñera Intestino	III	I	VI	A
4	58A	Vera	III	I	VI	A
4	59A	Pulmón Vera	III	I	VI V	A
5	56A	Pulmón Vera	IV	I	VI	B
6	53A	Pulmón Vera	III	I	VI	A
6	54A	Pulmón Vera	III	I	VI	A
6	55A		III	I	VI	A
6	57A		III	I		A
				I		

Blb"ogr.fí.

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